Prebiotics in fish production: effect on fish physiology and intestinal microbiota profile

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Nota Prévia

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Em todas as publicações decorrentes deste trabalho é devidamente referido que as instituições de origem da doutoranda Inês Maria dos Santos Guerreiro são:

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À minha família

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"If you cannot do great things, do small things in a great way."

Napoleon Hill

Abstract

Turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) are among the most intensively cultured marine fish species with high economical value in the Mediterranean region. White sea bream (*Diplodus sargus*) is still scarcely produced, but is presented as a worthy new species to diversify Mediterranean aquaculture production. For aquaculture to growth in a sustainable way some problems need to be surpassed, including the need to reduce the use of fisheries by-products in aquafeeds, improve fish growth and feed utilization, reduce disease incidence and the use of antibiotics. Prebiotics incorporation in aquafeeds appears as a good strategy for helping to achieve these goals. Despite the good results obtained in mammal nutrition, prebiotics such as short-chain fructooligosaccharides (scFOS), xylooligosaccharides (XOS), and galactooligosaccharides (GOS), are still poorly studied in fish. Moreover it is well known that prebiotics effect may change depending on several factors, such as rearing temperature and prebiotic dosage. Thus, the present work aimed at contributing to the knowledge of the effects of prebiotics, namely scFOS, XOS, and GOS in turbot, gilthead sea bream, European sea bass and white sea bream juveniles. To evaluate prebiotics effects a holistic approach was used, including the analysis of fish growth performance, feed utilization efficiency, whole-body composition, plasmatic metabolites, activities of key-enzymes of glycolytic, gluconeogenic, lipogenic, and amino acid metabolism, allochthonous gut microbiota, digestive enzymes activity, gut histomorphology, hepatic oxidative status, and immune response.

First (Chapter 2, Chapter 3 and Chapter 4) the effect of three levels of scFOS, 0.5, 1, and 2% included in diets with 50:50 of protein provided from fish meal (FM) and plant feedstuffs (PF) were tested in turbot of 32g reared at two temperatures, 15 and 20°C. No detectable differences were observed in fish immune status, and gut morphology. Also no differences were observed in gut microbiota composition, which may contribute to explain the lack of major effects on the other parameters analysed. Nonetheless, some scFOS effects were observed: 2% scFOS reduced the activities of malic enzyme and of glutamate dehydrogenase, and increased protein efficiency ratio. Dietary scFOS seemed to affect turbot's oxidative stress response, and the effects were dose and temperature related. Compared to the control diet, and in fish reared at 15°C, superoxide dismutase (SOD) activity was lower in fish fed 1% scFOS, while catalase (CAT) and glutathione reductase activities were lower in fish fed 0.5 and 1% scFOS. On the contrary, in fish reared at 20°C, SOD activity was higher in fish fed 1 and 2% scFOS, while CAT activity was lower in fish fed 0.5 and 2% scFOS, and glutathione peroxidase activity was lower in fish fed 2% scFOS but higher in fish fed 0.5% scFOS. Prebiotic effect on digestive enzymatic activities was also temperature and dosage related, and the differences were mainly related to dietary prebiotic levels rather than in relation to the control diet. Overall, results of this study indicate no major effects of dietary scFOS on turbot juveniles, at least at the tested levels.

On the following study (Chapter 5 and Chapter 6) the effect of three levels of scFOS, 0.1, 0.25, and 0.5%, were tested in gilthead sea bream of 32g fed diets with 50:50 of protein provided from FM and PF, and reared at two temperatures, 18 and 25°C. Aspartate aminotransferase activity was lower in fish fed 0.25% scFOS. Some minor effects were also observed in immune parameters: at 25°C, fish fed 0.1% scFOS had less lymphocytes, and fish fed 0.5% scFOS had lower total immunoglobulin than the other groups. At both temperatures, nitric oxide level was higher in fish fed 0.5% scFOS. No measurable effects were observed in gut bacterial composition, digestive enzymes activities, or gut morphology. Thus, as in turbot, no major effects of scFOS dietary supplementation were observed in gilthead sea bream juveniles on the measured parameters, at least at the tested levels.

From the above results in turbot and gilthead sea bream, is seems possible to conclude that rearing temperature interacts with scFOS effects, as different supplementation levels affected fish in different ways depending on temperature and prebiotic level.

In Chapter 7 and Chapter 8, the effect of 1% scFOS and of 1% XOS were studied in European sea bass of 60g fed FM-based diets (100% of the protein from FM) or PF-rich diets (30:70 of the protein from FM and PF, respectively). The effects of scFOS were limited, such as increased glucokinase activity in fish fed the FM-based diet, decreased SOD activity in fish fed the PF-rich diet, and increased activity of glucose 6-phosphate dehydrogenase in both cases. Independently of dietary protein source XOS decreased lipogenesis, improved growth performance in fish fed the PF-rich diet, and increased glycolytic activity in fish fed the FM-based diet. In fish fed both diets, XOS induced minor effects in gut morphology and in liver lipid peroxidation levels, but reduced hepatic antioxidant enzymatic activity. This indicates a positive effect of XOS on reduction of hepatic reactive oxygen species production. In conclusion, XOS seems to have good potential as prebiotic in European sea bass juveniles.

In the last study (Chapter 9 and Chapter 10) the effect of scFOS, XOS, and GOS, incorporated at 1% in diets with 30:70 of the protein from FM and PF, respectively, were evaluated in white sea bream of 53g. scFOS seemed to increase lipogenesis, while GOS ameliorated the adverse effects on intestinal histomorphology after 15 days of feeding the experimental diet. However, this effect disappeared by the end of the trial. As in European sea bass, XOS also decreased lipogenesis in white sea bream. In addition, XOS stimulated some parameters (alternative complement pathway, lysozyme, and total immunoglobulin) of the immune system, suggesting a possible enhanced immune status in fish fed this prebiotic. None of the prebiotics tested had measurable effects on fish gut microbiota or in hepatic oxidative status. Although in fish fed prebiotics some digestive enzymatic activities were increased at day 15, this effect was not observed by the end of the trial. In conclusion, of the three prebiotics tested in this study, XOS seems to be the most promising to be used in white sea bream juveniles diets.

Overall, our data indicates that prebiotic effects may be affected by rearing temperature and by dosage. Present data also suggest that scFOS and GOS seem to have little effect on the fish species studied, at least at the tested levels. Of the three prebiotics tested, XOS seems to be

the one with more potential to be used in PF-rich diets, and further studies are required to provide more detailed data of the potential of this prebiotic.

Keywords: Digestive enzymes; European sea bass; Gilthead sea bream; Gut histology; Gut microbiota; Immune status; Intermediary metabolism; Oxidative status; Prebiotics; Temperature; Turbot; White sea bream.

Sumário

O rodovalho (*Scophthalmus maximus*), a dourada (*Sparus aurata*) e o robalo (*Dicentrarchus labrax*) são das espécies mais produzidas em aquacultura e com maior valor económico na região Mediterrânica. O sargo (*Diplodus sargus*), embora ainda produzido em pequena escala, é sugerido como sendo uma espécie promissora para a diversificação da aquacultura nessa região. Para que a produção aquícola cresça de forma sustentável alguns problemas terão ainda de ser resolvidos. Entre os quais a redução do uso de farinhas de peixe nas rações, a melhoria do crescimento e eficiência alimentar nas espécies produzidas, e a diminuição da incidência de doenças sem o recurso a antibióticos. Os prebióticos surgem como uma alternativa com grande potencial para atingir os referidos objectivos. Alguns prebióticos, como é o caso dos fructooligossacarídeos de cadeia curta (scFOS), dos xilooligossacarídeos (XOS) ou dos galactooligossacarídeos (GOS) têm demonstrado produzir efeitos benéficos em mamíferos, mas ainda estão pouco estudados nos peixes. Os efeitos dos prebióticos podem ser afetados por diversos fatores, entre eles a temperatura da água usada na produção dos peixes ou as dosagens de incorporação dos prebióticos nas dietas. Assim, este trabalho teve como objetivo contribuir para o aumento do conhecimento do efeito dos prebióticos scFOS, XOS e GOS, em juvenis de rodovalho, dourada, robalo e sargo. Para esse efeito foram avaliados os seguintes parâmetros: crescimento, utilização do alimento, composição corporal, metabolitos plasmáticos, actividade de enzimas chave do metabolismo da glucose, dos lípidos e dos aminoácidos, microbiota intestinal alóctone, atividade de enzimas digestivas, histo-morfologia do intestino, estado oxidativo hepático e resposta imune.

No primeiro estudo (Capítulos 2, 3 e 4) foi avaliado o efeito da incorporação de scFOS a 0,5, 1 e 2% em dietas para rodovalho cuja proteína proveio 50:50 de farinha de peixe (FP) e de matérias-primas vegetais (MPV). O estudo foi realizado em rodovalhos de 32g cultivados a duas temperaturas, 15 e 20°C. O prebiótico não teve efeitos na comunidade bacteriana do intestino, o que pode explicar a ausência de efeitos noutros parâmetros analisados. Também não se observou qualquer efeito decorrente do uso do prebiótico na histo-morfologia do intestino ou no estado imune do peixe. De qualquer forma, o prebiótico afetou alguns parâmetros analisados: uma suplementação das dietas com 2% de scFOS levou a uma redução da atividade das enzimas málica e glutamato desidrogenase e a um aumento da eficácia de utilização proteica da dieta. O prebiótico parece também ter algum efeito na resposta oxidativa, mas os efeitos dependem da dosagem usada e da temperatura da água. Nos peixes mantidos a 15°C e alimentados com 1% de scFOS a enzima superóxido dismutase (SOD) teve uma atividade menor, enquanto que a atividade da catalase (CAT) e da glutationa redutase foi menor nos peixes alimentados com as dietas com 0.5 e 1% scFOS. Nos peixes mantidos a 20°C, a enzima SOD apresentou uma atividade maior nos peixes alimentados com 1 e 2% scFOS, enquanto que a CAT apresentou uma menor atividade nos peixes alimentados com 0.5 e 2% scFOS. Também a 20°C, e quando comparado com os peixes alimentados com a dieta controlo, a atividade da glutationa peroxidase foi menor nos peixes alimentados com 2% scFOS e maior

nos peixes alimentados com 0.5% scFOS. O efeito dos prebióticos na atividade das enzimas digestivas esteve dependente da temperatura da água e da dosagem do prebiótico. As diferenças registadas foram maioritariamente entre as diferentes dosagens do prebiótico e não em relação à dieta controlo. No geral, os resultados obtidos parecem indicar que não haverá um efeito significativo decorrente do uso de scFOS em rodovalho, pelo menos com os níveis testados.

No estudo seguinte (Capítulos 5 e 6) foi avaliado o efeito da incorporação de três níveis de scFOS: 0,1, 0,25 e 0,5% em dietas cuja proteína proveio 50:50 de FP e de MPV. O estudo foi realizado em douradas de 32g cultivadas a duas temperaturas, 18 e 25°C. A suplementação das dietas com 0,25% de scFOS causou uma redução na atividade da enzima aspartato aminotransferase. Alguns outros efeitos foram também observados nos parâmetros imunes, nomeadamente: a 25°C os peixes alimentados com 0,1% de scFOS tinham um menor número de linfócitos, e os peixes alimentados com 0,5% de scFOS tinham um menor nível de imunoglobulina total no plasma. Independentemente da temperatura, o óxido nítrico foi mais elevado nos peixes alimentados com a dieta suplementada com 0,5% de scFOS. A incorporação de scFOS nas dietas não afectou a comunidade bacteriana presente no intestino, a atividade das enzimas digestivas, e a histo-morfologia do intestino. Tal como no rodovalho, também em dourada não foram detetados efeitos significativos relacionados com a incorporação de scFOS nas dietas, pelo menos com os níveis testados.

Dos resultados obtidos em rodovalho e em dourada, podemos concluir que a temperatura interage com os efeitos do scFOS, já que os diferentes níveis de suplementação afetam os peixes de forma diferente dependendo da temperatura e do nível de suplementação das dietas com o prebiótico.

Nos Capítulos 7 e 8 foi testado em robalo (60g) o efeito de dois prebióticos, o scFOS e o XOS, que foram incorporados a 1% em dietas baseadas apenas em FP, ou com uma mistura de FP e MPV numa proporção de 30:70. A suplementação das dietas com scFOS levou a um aumento da atividade da enzima glucoquinase na dieta à base de FP, provocou um decréscimo na atividade da SOD na dieta com MPV, e aumentou a atividade da enzima glucose 6-fosfato desidrogenase em ambas as dietas. O prebiótico XOS causou um decréscimo na lipogénese com as duas dietas, melhorou o crescimento dos robalos alimentados com a dieta com MPV, e aumentou a atividade glicolítica nos robalos alimentados com dietas à base de FP. Para além disso, o XOS induziu apenas pequenas alterações na histo-morfologia do intestino e nos níveis de peroxidação lipídica do fígado, mas reduziu a atividade das enzimas antioxidantes com ambas as dietas. Isto sugere que o XOS contribuiu para a redução da produção de espécies oxigénio-reativas no fígado, o que leva a concluir que este prebiótico pode ter potencial para ser usado na produção de robalos.

No último ensaio, Capítulos 9 e 10, foram testados os efeitos em sargo (53g) de três prebióticos, scFOS, XOS e GOS. Os prebióticos foram incorporados a 1% em dietas ricas em MPV (30:70 da proteína proveniente de FP e MPV, respetivamente). A suplementação da dieta com scFOS pareceu aumentar a lipogénese. Após 15 dias de alimentação com a dieta com GOS

melhoraram os efeitos adversos no intestino causados pela alimentação com dietas ricas em MPV. No entanto, as diferenças entre dietas suplementadas ou não suplementadas com o prebiótico já não foram visíveis no final do ensaio. A suplementação da dieta com XOS levou a um decréscimo da lipogénese, tal como aconteceu no robalo. Além disso, estimulou alguns parâmetros (complemento, imunoglobulina total e lisozima) do sistema imune, sugerindo um possível reforço do estado imunológico dos animais. Nenhum dos prebióticos testados teve efeitos significativos no microbiota intestinal ou no estado oxidativo do fígado. Embora aos 15 dias após o início da alimentação com prebióticos a atividade enzimática de algumas enzimas digestivas estivesse aumentada, tal não foi observado no fim do ensaio. Concluindo, dos três prebióticos testados o XOS pareceu o mais promissor para ser usado em sargo.

No geral, os resultados desta Tese indicam que os efeitos dos prebióticos podem ser afetados pela temperatura de cultivo e pela dosagem usada. Os resultados indicam também que, pelo menos com os níveis testados e nas espécies estudadas, tanto o scFOS como o GOS parecem ter pouco efeito nos parâmetros estudados. Os resultados parecem sugerir que o XOS pode ter bom potencial para ser usado como prebiótico em peixes alimentados com dietas baseadas maioritariamente em MPV. Serão necessários mais estudos para fornecer dados mais detalhados sobre o potencial deste prebiótico.

Palavras-chave: Dourada; Enzimas digestivas; Estado imune; Estado oxidativo; Histologia do intestino; Metabolismo intermediário; Microbiota intestinal; Prebióticos; Robalo; Rodovalho; Sargo; Temperatura.

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Abbreviations List

ALA	Alpha-linolenic acid
ARA	Arachidonic acid
ASAT	Aspartate aminotransferase
AXOS	Arabinoxylooligosaccharides
cMOS	Concentrated MOS
COS	Chitosan
COS-REE	Chitosan oligosaccharide complex with rare earth
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DP	Degree of polymerization
EAA	Essential amino acids
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FE	Feed efficiency
FI	Feed intake
FM	Fish meal
FO	Fish oil
FOS	Fructooligosaccharides
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GOS	Galactooligosaccharides
HDL	High density lipoprotein
HUFA	Highly unsaturated fatty acids
IL	Interleukin
IMO	Isomaltooligosaccharides
LAB	Lactic acid bacteria
LC-PUFA	Long chain polyunsaturated fatty acids
LDL	Low density lipoprotein
MOS	Mannanoligosaccharides
N	Nitrogen
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
PER	Protein efficiency ratio
PF	Plant feedstuffs
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SBM	Soybean meal
SBO	Soybean oil
SCFAs	Short-chain fatty acids

scFOS	Short-chain fructooligosaccharides
TOS	Transgalactooligosaccharides
XOS	Xylooligosaccharides

Chapter 1
General Introduction

1.1 Aquaculture production

Aquatic animal production is the industry sector with the highest growth among animal production sectors. Between 1970 and 2010 it had an average annual growth rate of 2.9%, while total terrestrial meat production had an average annual growth rate of 2.7% (Tacon and Metian, 2013). In 2012, estimates for the world fisheries and aquaculture production were 158 million tonnes, from which 91.3 million tonnes were from fisheries and 66.6 million tonnes from aquaculture. Although the amount provided by captures is higher than that from aquaculture, aquaculture production is steadily increasing every year, while capture production has almost stabilised in the last 20-30 years (Figure 1) (FAO, 2014).

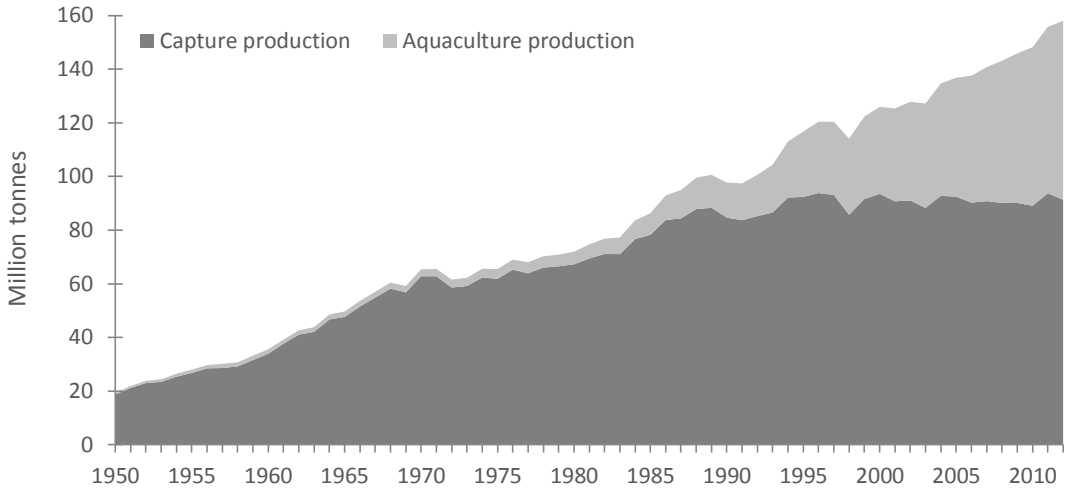

Figure 1 – World capture fisheries and aquaculture production between 1950 and 2012
Adapted from FAO (2014)

It is important that aquaculture production continues growing to fulfil the increasing human needs for food fish and to replace capture fisheries, at least to the level that allows the overexploited fisheries stocks to be replenished. Thus, nowadays aquaculture assumes a great importance in meeting food and nutrition requirements of a growing human population. Fish, compared with farm animals, is a better source of high quality protein, micronutrients, particularly phosphorus, selenium and iron, and of essential fatty acids (EFA), especially long chain polyunsaturated fatty acids (LC-PUFA) (FAO, 2014; Tacon and Metian, 2013).

Aquaculture production in the Mediterranean region began many centuries ago as extensive rearing in ponds and coastal lagoons, and evolving to the currently high intensive raceways or cage fish farm exploitations. The proportion of marine fish in overall Mediterranean aquaculture output has increased greatly, from 13% in 1995 to 36% in 2007. Egypt, France, Spain, Italy, Turkey and Greece are the main producing countries in the Mediterranean region. Production became focused almost exclusively on high value species such as turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus aurata*), and European sea bass (*Dicentrarchus labrax*). In fact, it is the commercial culture of those species that led to the huge growth production observed in the Mediterranean aquaculture industry over the last two

decades. Among Sparidae, white sea bream (*Diplodus sargus*), arises as a new species with high interest for the diversification of Mediterranean aquaculture, however it still has a limited production (Barazi-Yeroulanos, 2010).

1.1.1 Aquafeeds – Fish meal vs Plant feedstuffs

Feeds represent a huge part of aquaculture production costs. Thus, nutritional balanced diets are essential for proper development of aquaculture, as only by correct feeding with nutritionally adequate diets is possible to economically produce a high quality product at competitive costs.

Aquaculture production of carnivorous species relies heavily in fish meal (FM) and fish oil, respectively as main protein and lipid sources for aquafeeds. Due to the depletion of wild stocks and consequent rise in prices, sustainable alternative ingredients are needed (Tacon et al., 2011). Moreover, the use of alternative ingredients allow to surpass problems inherent to the use of fish by-products, such as organic and inorganic contaminants, shortage of supply, environment sustainability, and net effect of demand-and-supply economics (Gatlin III et al., 2007).

Alternatives to FM are, for instance, land animal protein meals, plant feedstuffs (PF) or microbial protein sources (Tacon et al., 2011). However, to be sustainable alternatives to FM, ingredients must fulfil some practical characteristics, such as wide availability, competitive price, ease of handling, shipping, storage, and incorporation in feed production. Besides these practical characteristics, alternative feed ingredients should also meet some nutritional characteristics, such as having low levels of fibre, starch, non-starch carbohydrates, and antinutrients, have fairly high protein content and adequate amino acid profile, high nutrient digestibility, and reasonable palatability (Gatlin III et al., 2007).

PF are nowadays the most used alternatives to FM in aquafeeds (Tacon et al., 2011). Ingredients such as soy protein concentrate, corn or wheat gluten were pointed out as having most of the above mentioned characteristics, yet with the drawback of being expensive (Gatlin III et al., 2007). However, most of the PF present disadvantages such as having relatively low-protein content, amino acid imbalances, low palatability, presence of endogenous antinutritional factors, and large amounts of carbohydrates, namely non-starch polysaccharides (Gatlin III et al., 2007). Nonetheless, strategies were developed to overcome some of those disadvantages. Problems with low protein content, amino acid imbalances or low palatability issues are surpassed by using complementary protein sources and plant protein concentrates, supplementing with the limiting amino acids, and using feed attractants (Davies et al., 1997; Dias et al., 1997; Gómez-Requeni et al., 2003; Watanabe et al., 2001; Zhang et al., 2012).

Still, the main limitations related to the use of PF are the antinutritional factors. While some antinutritional factors are heat-labile and thus easily removed during processing, others are heat-resistant and consequently more difficult to remove. Extraction with water or addition of

feed supplements can also be used to overcome antinutritional factor issues (Francis et al., 2001). Moreover, depending of the PF used, several antinutritional factors may be present making it difficult to determine which factor is causing the specific adverse effects (Francis et al., 2001; Gatlin III et al., 2007). Among possible harmful effects of antinutritional factors, are reduced palatability, reduced nutrients utilization, altered nutrient balances in the diets, reduced growth, intestinal dysfunctions, altered gut microbiota and immune modulation, hypoglycaemia, and liver damage (Krogdahl et al., 2010). The intestinal damages usually reported in fish fed PF, namely soybean containing diets, are enteritis-like changes, such as decrease or absence of absorptive vacuoles, shortening mucosal folding heights, and profound infiltration of lamina propria inflammatory cells (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2000; van den Ingh et al., 1991). These soybean related effects are particularly important in salmonids, while in species such as the European sea bass and gilthead sea bream such negative effects are less evident (Couto et al., 2014a, 2015).

1.1.2 Aquafeeds - Functional Ingredients

A functional ingredient may be or not a nutrient, but has a physiologic effect beyond the traditional nutritional effect, and affects one or more functions in the body, improving health or disease resistance (Roberfroid, 2000). The incorporation of functional ingredients such as probiotics and prebiotics were suggested as a possible strategy for enhancing the utilization of PF in aquafeeds (Gatlin III et al., 2007).

Moreover, intensification of aquaculture production may lead to outbreaks of a variety of infectious diseases leading to heavy losses and hinder production. Therefore, improving and protecting fish health is a major concern in fish farms and until recently this was mainly achieved by applying antibiotics. However, the use of antibiotics in the last decades has been severely criticized and the EU moratorium (Regulation (EC) No 1831/2003) banned its use as growth promoters in animal feeds (Regulation, 2003). Besides, the use of antibiotics may enhance the development of antibiotic resistant bacteria, leave residues in the seafood, damage microbial communities in the aquatic environment, and suppress fish immune system (Capone et al., 1996; Collier and Pinn, 1998; Sapkota et al., 2008). To avoid these inconveniences, functional ingredients such as probiotics and prebiotics have become to be used in aquaculture as an alternative to antibiotics (Dimitroglou et al., 2011a; Ringø et al., 2010).

Improvements in fish performance, health and disease resistance when administering some functional ingredients is mostly connected with changes in gut bacterial communities. However, contrary to gut microbial communities of homoeothermic animals that thrive under fairly constant conditions, fish gut microbial communities are constantly subjected to important variations of the aqueous habitat, which may considerably change regarding temperature, salinity, and surrounding bacterial composition (Merrifield and Rodiles, 2015). Thus, gut bacterial communities assume great importance in fish, as shown in gnotobiotic

zebrafish (*Danio rerio*) where the presence of 212 host genes regulated by gut microbiota where revealed (Rawls et al., 2004). Among those genes, some were involved in processes related to immunity, nutrition, cell division, or DNA replication (Rawls et al., 2004). Accordingly, gut microbiota affects the host in several ways, including development, digestion, nutrition, disease resistance, and immunity (Romero et al., 2014). Bacteria assumes such importance in fish gut development that zebrafish reared in a germ-free environment fails to develop correctly. For instance, fish lack brush border intestinal alkaline phosphatase activity, present immature patterns of glycan expression, and paucity of goblet and enteroendocrine cells. In addition, gut fails to take up protein macromolecules in the distal gut and exhibit faster motility (Bates et al., 2006).

Fish microbial community comprises viruses, Archaea, protozoa, yeasts, and bacteria. In terms of abundance, bacteria are typically the dominant microbes present in the gut and may comprise from hundreds to thousands of operational taxonomic units, present in levels of up to log 11 cells per gram of faecal material. Species of the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Tenericutes* are reported among the most dominant members present in fish gut (Merrifield and Rodiles, 2015). Gut microbial community can be divided in two groups: allochthonous microbiota, which is the group that passes through the gut with the feed or digesta, and autochthonous microbiota, which is closely associated with the host tissues and, at least in theory, is resident (Romero et al., 2014).

Probiotics are one of the most known and used functional feed ingredients, and are strictly connected with gut microbiota. First definitions of probiotics appeared on the 1960s and 1970s and were imprecise, the first generally accepted definition was the one proposed by Fuller (1989) in the 1980s, which stated that a probiotic is “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. Recently, Merrifield et al. (2010) proposed a new definition, which merged all previous definitions and adapting it better to the aquaculture context. According to Merrifield et al. (2010) a probiotic is “a live, dead or component of a microbial cell that when administered via the feed or to the rearing water benefits the host by improving either disease resistance, health status, growth performance, feed utilisation, stress response or general vigour, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment.”

To be classified as probiotic, a probiont needs to fulfil several characteristics, such as: not being pathogenic for the host, environment and consumers; being free of plasmid-encoded antibiotic resistance genes; being resistant to bile salts and the low pH of the gastrointestinal (GI) tract (Merrifield et al., 2010). Moreover, some extra characteristics might be favourable, such as: the probiont should be able to adhere and growth in the host gut; be registered for use as a feed additive; display advantageous growth characteristics; produce relevant extracellular digestive enzymes or vitamins; be indigenous to the host or the rearing

environment; remain viable under normal storage conditions; be robust enough to survive feed processing (Merrifield et al., 2010).

The most common mechanisms of action of probiotics consist in competitive exclusion of pathogenic bacteria, enhancement of host nutrition, enzymatically contribute to nutrients digestion, and stimulate host immune response (Pérez-Sánchez et al., 2014). Beneficial effects reported for probiotics in fish include improved growth performance, carcass composition, feed utilisation, digestive enzyme activities, health status, disease resistance, antioxidant status, gut morphology, gut microbial composition, and reduced stress and malformations (Dimitroglou et al., 2011a; Merrifield et al., 2010; Pérez-Sánchez et al., 2014).

Despite all potential advantages of probiotics, some problems might be inherent to its use. Probiotics are mainly live organisms (generally bacteria) that can change the surrounding environmental bacterial communities and, without further advances in feed technology, do not withstand extrusion conditions. In addition, it is difficult to keep a constant probiotic level in fish feeds, and these feeds have short shelf-life (Dimitroglou et al., 2011a; Lauzon et al., 2014; Merrifield et al., 2010). Therefore, prebiotics appeared as an environmental friendly alternative to both antibiotics and probiotics.

1.2 Prebiotics as functional ingredients

The term prebiotic was first introduced by Gibson and Roberfroid (1995) exchanging the prefix “pro” for “pre”, meaning “before” or “for” (Schrezenmeir and de Vrese, 2001). The proposed definition of prebiotics is: “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health” (Gibson and Roberfroid, 1995).

To be classified as prebiotic, the following characteristics need to be satisfied (Gibson and Roberfroid, 1995):

1) it cannot be hydrolysed and absorbed in the upper part of the GI tract;
2) it needs to stimulate specific beneficial bacteria commensal to the host GI tract, stimulating their growth and/or activity;
3) it must be able to change GI microbiota in favour of a healthier composition;
4) it needs to induce luminal or systemic effects that benefit host health.

Therefore, contrary to probiotics where a beneficial bacteria is introduced in the diet or the water, prebiotics supplemented to diets will selectively stimulate the growth of specific autochthonous beneficial bacteria (Dimitroglou et al., 2011a).

After this first classification several feed components were named prebiotics, without due consideration to the criteria required. Therefore, Gibson et al. (2004) stated that a clear criteria needed to be established, and that the classification of an ingredient as prebiotic required scientific demonstration that it:

1) resists gastric acidity, hydrolysis by mammalian enzymes, and GI absorption;
2) is fermented by intestinal microflora;
3) selectively stimulates the growth and/or activity of intestinal bacteria associated with health and wellbeing.

Of the above three points, the last one is the most important and at the same time the most difficult to demonstrate, since it requires reliable and quantitative analysis of total aerobic and anaerobic bacteria, and of specific groups such as: *Bifidobacterium*, *Lactobacillus* and Enterobacteria (Gibson et al., 2004).

Accordingly, Gibson et al. (2004) stated that only three oligosaccharides fulfilled the three criteria: inulin, transgalactooligosaccharides (TOS) and lactulose. Since then, several other oligosaccharides were considered as prebiotics for fish: mannanoligosaccharides (MOS), fructooligosaccharides (FOS, or oligofructose), short-chain fructooligosaccharides (scFOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), and some commercial mixtures of prebiotics. Of the above, the most studied prebiotics in fish are inulin, MOS, and FOS (Dimitroglou et al., 2011a; Ringø et al., 2010).

These oligosaccharides are called prebiotics considering that the term prebiotic applies to oligosaccharides that potentially meet the criteria for prebiotic classification, rather than restricted to oligosaccharides that have been definitively proven to meet all criteria (Lauzon et al., 2014). The main prebiotics currently available as feed additives are made of monosaccharides units as fructose, galactose, glucose, or xylose (Figure 2).

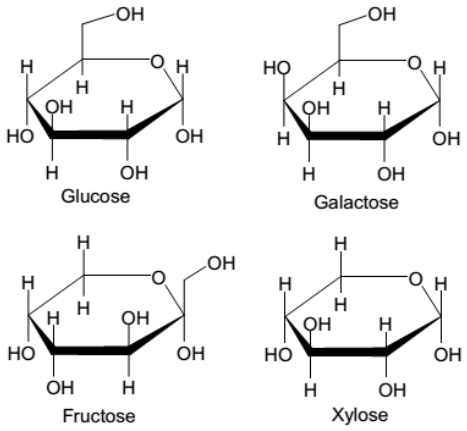

Figure 2 – Monosaccharides components of prebiotics
(Mussatto and Mancilha, 2007)

Prebiotics are not digested by the host, but are fermented by bacteria present in the host gut, such as *Bifidobacterium* and *Lactobacillus*. Bacteria fermentation leads to the production of short-chain fatty acids (SCFAs) such as acetic, propionic, and butyric acids, which cause a pH drop, lead to colonic and systemic health effects, and might be absorbed by the host and used as energy sources (Figure 3).

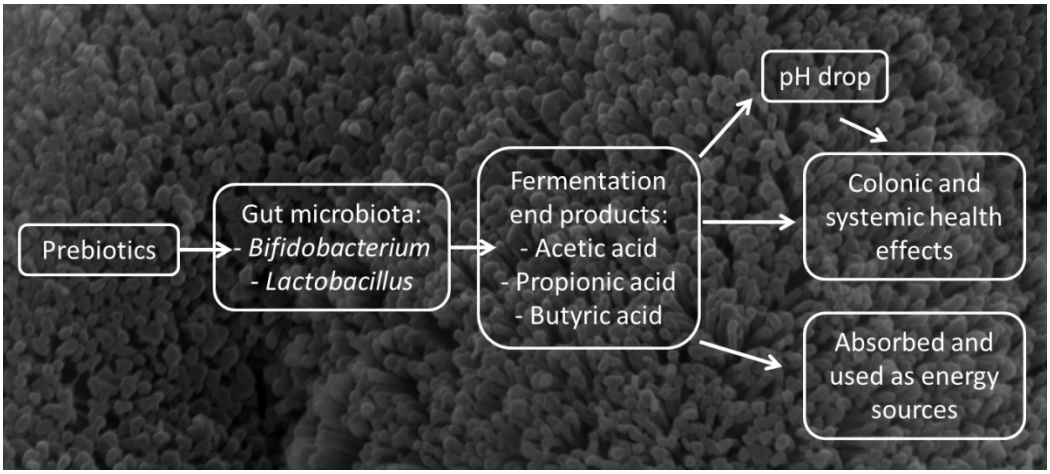

Figure 3 – Prebiotics action in the gastrointestinal tract
Adapted from Huazano-García and López (2013)

1.2.1 Prebiotics benefits and possible modes of action

The reported benefits of prebiotics on fish are many; for instance, improved growth performance, feed utilization, carcass composition, health status, disease resistance, gut morphology, and microbiota modulation (Dimitroglou et al., 2011a; Merrifield et al., 2010; Ringø et al., 2010; Ringø et al., 2014). Other benefits such as improved glucose and lipid metabolism or reduced oxidative stress are reported in mammals and remain understudied in fish (Delzenne, 2003; Gibson and Roberfroid, 1995; Gobinath et al., 2010; Wang et al., 2011). However, the specific modes of action that promote the observed host benefits are often difficult to completely elucidate, due to multiple modes of action and synergies that might occur. Therefore, much of the knowledge available about the possible modes of action of prebiotics is derived from mammal's studies. Nonetheless, suggested modes of action of prebiotics in fish include changes in bacterial communities resulting in an increase of beneficial communities that promote the production of inhibitory compounds, competition for chemicals or for available energy, competition for adhesion sites, inhibition of virulent gene expression or disruption of quorum sensing (Merrifield et al., 2010). Other modes of action related to prebiotics include the bacterial end-products of fermentation (Delzenne, 2003; Gibson and Roberfroid, 1995), or the interaction between prebiotics and pattern recognition receptors (Song et al., 2014; Torrecillas et al., 2014).

1.2.1.1 Gut microbiota

According to the definition of prebiotics, which state that prebiotics beneficially affect the host by selectively stimulating growth or activity of specific bacteria present in the gut (Gibson and Roberfroid, 1995), it is clear that the primary effects of prebiotics are on gut microbiota.

Several studies of prebiotics effects on gut microbiota were based on culture-dependent analysis, where aerobic heterotrophic bacteria are determined by plate counts (Akrami et al., 2013; Dimitroglou et al., 2009; Hoseinifar et al., 2011b; Hoseinifar et al., 2013; Hoseinifar et al.,

2014a; Hoseinifar et al., 2014b; Hoseinifar et al., 2015a; Hui-Yuan et al., 2007; Mahious et al., 2006; Ortiz et al., 2013; Zhou et al., 2007). However, this method presents limitations since the number and species of bacteria detected are affected by the culture conditions and media, especially in the case of fastidious and obligate anaerobic bacteria. In addition, culture dependent methods are time consuming and lack accuracy in isolates identification. In fact, it appears that cultivable bacteria may represent less than 1% of total bacteria present in the gut (Merrifield and Rodiles, 2015; Romero et al., 2014).

To overcome these limitations, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) begun to be used (Burr et al., 2008b, 2010; Cerezuela et al., 2013a; Dimitroglou et al., 2009; Dimitroglou et al., 2010b; Li et al., 2007; Raggi and Gatlin III, 2012; Torrecillas et al., 2012; Zhou et al., 2009). DGGE is a relatively inexpensive and easy to perform technique, providing a fast method for assessing gut microbiota. Contrary to the less than 1% of bacteria identified by culture dependent methods, DGGE may detect 90-99% of the bacterial community. DGGE specificity is based on the electrophoresis of targeted regions of 16S RNA gene, typically the V3 region, which displays differing denaturing properties depending on nucleotide composition (Zhou et al., 2014). Today, besides DGGE, other molecular techniques are also being used, due to their culture independent nature and quantitative results, such as fluorescent in situ hybridization, quantitative real-time PCR, histochemistry, enzyme-linked immunosorbent assay, or community libraries using next-generation sequencing (Zhou et al., 2014).

To this point, it is clear that prebiotic mode of action on bacterial gut communities is mainly through its fermentation by specific beneficial bacteria (such as *Lactobacillus*, and *Bifidobacterium*), which possess the necessary enzymes to digest prebiotics and thus are favoured relatively to less beneficial bacteria. The SCFAs produced as end-products of fermentation are then responsible for the many reported beneficial effects of feeding animals with prebiotics (Dimitroglou et al., 2011a; Gibson et al., 2004; Merrifield et al., 2010; Ringø et al., 2010; Roberfroid et al., 2010). In fact, several studies reported an increase in LAB counts in fish fed prebiotics (Hoseinifar et al., 2011b; Hoseinifar et al., 2013; Hoseinifar et al., 2014a; Hoseinifar et al., 2014b; Hoseinifar et al., 2015a). Besides their use by beneficial bacteria, prebiotics such as MOS mimic specific carbohydrate groups of enterocytes and, therefore, favour pathogenic bacterial adhesion to the prebiotic rather than to the enterocytes. These pathogenic bacteria associated to the prebiotic are then removed with faeces. This mechanism of action reduces the incidence and severity of potential diseases (Torrecillas et al., 2014).

1.2.1.2 Growth performance

One well studied aspect regarding prebiotics effect in fish is their effect on growth performance and feed utilization (Dimitroglou et al., 2011a; Merrifield et al., 2010; Ringø et al., 2010; Ringø et al., 2014). The enhanced feed efficiency (FE), nutrient digestibility and growth improvements associated with dietary prebiotics may be due to changes in digestive enzymes or in gut morphology. Anguiano et al. (2013) studied the digestive enzymes and gut

histomorphology of red drum (*Sciaenops ocellatus*) fed FOS, MOS, TOS, and GroBiotic®-A, and of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) fed GroBiotic®-A. The authors concluded that the observed improvement of nutrient digestibility in response to dietary prebiotic supplementation was more likely related with changes in gut structure than with improvements on digestive enzyme activities. On the other hand, the higher growth of allogynogenetic crucian carp (*Carassius auratus gibelio*), Caspian roach (*Rutilus rutilus*), and blunt snout bream (*Megalobrama amblycephala*) when fed prebiotics was well related with increased activities of digestive enzymes (Soleimani et al., 2012; Wu et al., 2013; Xu et al., 2009). In any case, the observed results may change among studies since prebiotic effects may change due to several factors, such as prebiotic source, supplementation level, fish species and age, rearing conditions and diet composition. In fact, results with different species and prebiotics are still confusing, since some studies report growth beneficial effects (Hoseinifar et al., 2013; Li et al., 2008; Soleimani et al., 2012; Wu et al., 2013; Xu et al., 2009; Zhou et al., 2010), while others studies report lack of effects (Buentello et al., 2010; Burr et al., 2010; Grisdale-Helland et al., 2008; Hoseinifar et al., 2014a), or even a tendency for negative effects with high prebiotic levels (Hoseinifar et al., 2011b).

1.2.1.3 Intermediary metabolism

It is well known that prebiotics affect glucose and lipid metabolism in mammals (Delzenne, 2003; Roberfroid et al., 2010). Studies indicated that prebiotics may reduce hepatic lipogenesis, serum and liver cholesterol and triglycerides levels, increase serum high density lipoprotein/low density lipoprotein (HDL/LDL) ratio, and have a protective effect against steatosis (Delzenne, 2003; Delzenne et al., 2008; Roberfroid et al., 2010; Teitelbaum, 2009). Prebiotics can also improve glucose tolerance, by lowering plasma glucose levels and enhancing insulin sensitivity (Delzenne, 2003; Delzenne et al., 2008; Roberfroid et al., 2010; Teitelbaum, 2009).

Studies on the effect of prebiotics on amino acid metabolism are scarce. One study in cats fed a mixture of oligofructose and inulin reported a reduction in plasmatic aspartate aminotransferase (ASAT) activity (Verbrugghe et al., 2009). In that study several factors were advanced for an amino acids sparing effect: the decrease in ASAT activity suggested an inhibition of gluconeogenesis from aspartate; increased propionylcarnitine concentration indicated an inhibition of gluconeogenesis from pyruvate, also resulting in amino acids sparing; a trend for decreased methylmalonylcarnitine also supports the reduction of amino acid catabolism, since it is known this molecule is a metabolite of valine, methionine, and isoleucine catabolism. Thus, inhibition of amino acid catabolism due to dietary incorporation of prebiotics seems to be mainly due to an enhancement of gluconeogenesis from propionate (Verbrugghe et al., 2009).

No studies are available in fish regarding prebiotics effects on amino acid or glucose metabolism. Prebiotic effects were only studied on lipid metabolism, and the available

information is still very scarce (Torrecillas et al., 2011b; Torrecillas et al., 2015b). In accordance with results reported in mammals, feeding MOS to European sea bass led to a decrease in the activity of key enzymes of lipogenesis, namely malic enzyme and glucose-6-phosphate dehydrogenase activities (Torrecillas et al., 2011b). Nevertheless, the prebiotic lowering effect on plasmatic glucose, cholesterol, and triglycerides, which were reported in mammals are not evident in fish. Ye et al. (2011) reported reduced triglycerides and cholesterol-LDL in Japanese flounder (*Paralichthys olivaceus*) fed FOS, while other authors report no changes in these plasmatic metabolites in beluga (*Huso huso*) (Reza et al., 2009). Hoseinifar et al. (2011a) also reported no changes in plasma glucose, but observed lower plasma cholesterol (only with 2% oligofructose) also in beluga.

Studies with rats reported a reduction of fat deposition due to dietary FOS (Gibson and Roberfroid, 1995). In European sea bass Torrecillas et al. (2011b) also observed lower body lipids in fish fed with 0.6% MOS, but most studies in fish fed FOS, MOS, GOS or inulin reported no effects on body lipid (Buentello et al., 2010; Burr et al., 2010; Dimitroglou et al., 2010b; Dimitroglou et al., 2011b; Grisdale-Helland et al., 2008; Hoseinifar et al., 2011b; Hoseinifar et al., 2013; Reza et al., 2009; Ye et al., 2011), or even an increase of body lipids with scFOS and FOS (Hoseinifar et al., 2015a; Wu et al., 2013; Zhang et al., 2015).

The modulation of glucose metabolism reported in mammals fed prebiotics is related to the colonic production of SCFAs, mostly acetate and propionate, generated through prebiotics fermentation by GI microbiota (Delzenne, 2003; Roberfroid et al., 2010). Thus, in isolated rat hepatocytes propionate stimulated glycolysis whereas the opposite was observed with acetate and butyrate (Anderson and Bridges, 1984). The enhancement of glycolysis with propionate was related with a decrease of hepatic citrate concentration, which is a metabolic inhibitor of phosphofructokinase, the enzyme that phosphorylates fructose 6-phosphate in glycolysis (Blair et al., 1973). Besides stimulating glycolysis, propionate also decreased gluconeogenesis in mammals via inhibition of pyruvate carboxylase, which catalyze the conversion of pyruvate to oxaloacetate (Anderson and Bridges, 1984; Blair et al., 1973). In contrast, acetate and butyrate increased glucose production from lactate in rat isolated hepatocytes (Anderson and Bridges, 1984). Also in mammals, propionate stimulated the production of the intestinal hormone glucagon-like peptide 1, which in turn stimulated insulin secretion, leading to an increase of liver glycogen synthesis and a decrease of plasma glucose levels (Cani et al., 2005; Delzenne et al., 2007; Frost et al., 2003).

The modulation of lipid metabolism by the main SCFAs, namely acetate and propionate, is converse. Thus, whereas acetate is a lipogenic substrate, propionate is a competitive inhibitor of the entrance of acetate into liver cells (Delzenne et al., 2008; Teitelbaum, 2009). Propionate was also reported to inhibit lipid synthesis in rat hepatocytes (Nishina and Freedland, 1990; Wright et al., 1990). Moreover, acetate supplied in the diet of diabetic mice at a dose of 0.3% activates liver 5' adenosine monophosphate-activated protein kinase, an enzyme related with the inhibition of lipogenesis (Sakakibara et al., 2006). Besides the effects of SCFAs produced by prebiotic fermentation, a direct increase of bacteria such as lactic acid bacteria (LAB) may also

affect lipogenesis. Park et al. (2013) reported that a mixture of two *Lactobacillus* sp. lead to down-regulation of sterol regulatory element-binding protein-1, fatty acid synthase, and stearoyl-CoA desaturase-1 expression, reflecting suppression of lipogenesis in rats.

Consequently, studies that evaluate gut microbiota and SCFAs production are needed to understand the mechanism by which these end-products of fermentation may affect lipid and glucose metabolism in fish. Improved lipid metabolism may contribute to produce leaner fish, which meets consumers' preference when compared with wild fish. Increased glucose tolerance may allow higher incorporation of carbohydrates in the diets, thus allowing higher inclusion of PF in fish diets. Moreover, the potential protein sparing by dietary prebiotics is also worthy of further studies.

1.2.1.4 Oxidative status

When reactive oxygen species (ROS) production is higher than ROS removal oxidative stress occurs. Fish, as other aerobic organisms are also susceptible to ROS attack and developed antioxidant defences based on substances such as vitamins C, and E, uric acid, glutathione, and carotenoids, besides diverse antioxidant enzymes (Martinez-Alvarez et al., 2005; Storey, 1996).

Prebiotics were reported as positively affecting ROS generation in fish, by decreasing oxidative damage or increasing antioxidant potential (Zhang et al., 2013; Zhang et al., 2014). Prebiotics such as inulin were even reported as antioxidants, with ROS scavenging ability (Stoyanova et al., 2011; Van den Ende et al., 2011). The antioxidant activity mechanisms of prebiotics are yet to be completely clarified for some prebiotics, such as FOS (Pejin et al., 2014; Zhang et al., 2013) or GOS, which even in mammals were scarcely studied regarding their effects on oxidative stress (Malardé et al., 2015).

One possible mode of action of prebiotics on stress oxidative levels is through SCFAs, which may have a role in oxidative stress modulation. In fact, butyrate, more than other SCFA, was reported as being related with a significant reduction of hydrogen peroxide induced DNA damage in rats and humans (Abrahamse et al., 1999; Rosignoli et al., 2001; Toden et al., 2007). Some probiotic strains of the LAB clade were also reported to possess anti-oxidative activity. For instance, rats fed a mixture of two *Lactobacillus* sp. were reported to increase β-oxidation via up-regulation of peroxisome proliferator-activated receptor α and carnitine palmitoyltransferase 2 mRNA levels (Park et al., 2013). Other possibility may be connected with prebiotic composition. For instance, XOS has ferulic acid in its composition, which was reported as having a very strong antioxidant activity mainly due to its phenolic nucleus and extended side-chain conjugation that readily forms stabilised phenoxy radicals and terminate chain reactions (Graf, 1992). In fact, ferulic acid scavenges superoxide anion radicals in a way similar to superoxide dismutase, an important antioxidant enzyme (Toda et al., 1991).

In fish, the antioxidant potential of prebiotics, namely FOS, was related with its bifidogenic effect, since helping gut microbial defence mechanisms may help surpassing both exogenous and endogenous oxidative stress. Moreover, prebiotics may have a role in translation and

post-translational process of antioxidant enzymes (Zhang et al., 2013; Zhang et al., 2014). However, both hypothesis still need to be confirmed.

1.2.1.5 Immune response

Dietary prebiotic supplementation is generally reported to enhance immune status in fish, which were shown to present increased immune parameters such as white blood cell counts, lysozyme, alternative complement, and immunoglobulins (Akrami et al., 2013; Buentello et al., 2010; Cerezuela et al., 2012; Geraylou et al., 2012; Li et al., 2008; Soleimani et al., 2012).

Once in the gut, prebiotics contact with gut-associated lymphoid tissue (GALT), which in fish is formed by intraepithelial and lamina propria leucocytes, including B and T lymphocytes, macrophages, and eosinophilic and neutrophilic granulocytes (Rombout et al., 2011). This physical barrier, composed by epithelia and their mucus secretions, is an integrant and essential part of the fish immune system. It represents one of the first lines of defence, as gut is one of the first entering routs of fish pathogens in the organism (Hoseinifar et al., 2015b).

Song et al. (2014) stated that prebiotics activate the host innate immune system in two ways: by directly stimulating the innate immune system, or by enhancing the growth of commensal microbiota. Hoseinifar et al. (2015b) further stated that although the mechanisms beyond prebiotic systemic immunity enhancement in fish remain unknown, several hypothesis may be advanced to explain it: GALT leucocytes can directly contact with the luminal prebiotics and be activated; enterocytes metabolism, which are physiologically and morphologically changed by prebiotics, may mediate immune activation, through the production of factors that increase leucocytes recruitment and functions; microbiota changes due to prebiotics may be, per se or by their own metabolic products, responsible for the immune activation; prebiotics may cross the epithelia and encounter and activate leucocytes present in the epithelium. Thus, prebiotics may directly interact with pattern recognition receptors, such as β-glucan receptors and dectin-1 receptors, that are expressed on macrophages (Song et al., 2014). For instance, fish head kidney leucocytes were described as having a mannose receptor, which may explain the enhanced phagocytic activity (Torrecillas et al., 2011b).

The immunostimulatory nature of prebiotics, may as well be a direct effect of growth stimulation of gut beneficial bacteria such as *Lactobacillus* sp., *Bifidobacterium* sp., and *Bacillus* sp. (Broekaert et al., 2011; Buentello et al., 2010; Geraylou et al., 2012; Song et al., 2014). These bacteria have in their composition substances such as peptidoglycans, which have been reported to increase innate defence mechanisms in rainbow trout (*Oncorhynchus mykiss*) (Bricknell and Dalmo, 2005). As other example, Peyer's patch cells cultivated with *Bifidobacterium breve* presented augmented production of anti-inflammatory cytokines and immunoglobulins and enhanced macrophage phagocytic activity (Yasui and Ohwaki, 1991). Additionally, SCFAs produced during prebiotic fermentation may be taken up directly by the fish and enhance its immune status, as suggested for Siberian sturgeon (*Acipenser baerii*) fed AXOS (Geraylou et al., 2012). In fact, cell-culture studies demonstrated that butyrate inhibits

pro-inflammatory interleukin-2 (IL) and interferon γ production, and acetate and propionate increase immuno-regulatory IL-10 production (Roberfroid et al., 2010).

The anti-pathogenic activity of prebiotics can be illustrated by GOS, which in mammals was shown to prevent bacterial attachment to colonic epithelium. GOS contains structures similar to the glycoconjugates of glycoproteins and lipids present on the microvillus membranes, and thus it may interfere with bacterial receptors by binding to them (Sangwan et al., 2011).

An enhanced immune status leads to an improved immune response, which may be reflected in higher survival of fish challenged with bacteria or extreme environmental conditions (Hoseinifar et al., 2013; Hoseinifar et al., 2014b; Hoseinifar et al., 2015c; Soleimani et al., 2012).

1.2.1.6 Gut morphology

Prebiotics use in fish were reported to improve gut morphology, by increasing gut absorptive area, microvilli density and height, and villi structure complexity (Dimitroglou et al., 2009; Dimitroglou et al., 2010b; Dimitroglou et al., 2011b; Zhou et al., 2010). Gut morphology improvement directly affects fish immunological status and, consequently, fish health, as preservation of a healthy mucosal epithelium reduces the odds of opportunistic indigenous bacterial infections (Dimitroglou et al., 2011b). These improvements in gut morphology may also explain the reported growth increase when prebiotics are fed to fish (Anguiano et al., 2013; Soleimani et al., 2012).

Improvement in gut structure may be related with the end-products of prebiotic fermentation, since SCFAs are absorbed and metabolized by the enterocytes, thus accounting for a large proportion of enterocytes energy needs, and stimulate the growth of gut beneficial bacteria, including LAB, which may also help maintaining gut homeostasis (Merrifield et al., 2010; Mountfort et al., 2002).

However, caution should be taken when evaluating results, since prebiotic effects can change depending on factors such as fish age or the method used to analyse gut histomorphology. Dimitroglou et al. (2010b) fed FM and soybean meal-based (SBM) diets supplemented with MOS to gilthead sea bream and observed that the prebiotic had no effect on mucosal folds morphology of the anterior gut. However, MOS appeared to improve the absorptive surface area in the posterior gut of fish fed the FM diet, as denoted by higher perimeter of the intestinal lumen. However, using electron microscopy techniques, it became evident that in both fish fed FM and SBM-based diets MOS affected both anterior and posterior gut at the ultrastructural level. In another study, Dimitroglou et al. (2009) showed that gut histology in fish fed MOS may change depending on fish age. The authors observed improvements in gut morphology, such as increased absorptive surface, microvilli density and length, of sub-adult rainbow trout fed MOS, whereas no effects were observed in trout juveniles.

1.2.1.7 Digestive enzymes

Assuming that prebiotics modulate gut microbiota (Dimitroglou et al., 2011a; Merrifield et al., 2010; Ringø et al., 2014), and accepting that gut microbiota has an important role in aiding host digestion (Merrifield and Rodiles, 2015), it can also be assumed that prebiotics have beneficial effects on digestive enzymes activities.

Thus, the increased digestive enzymatic activities in fish fed prebiotics may, at least in part be due to bacterial digestive enzymes production. In fact, *Bacillus* sp., *Enterobacteriaceae*, *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Photobacterium*, *Pseudomonas*, Vibrio, *Microbacterium*, *Micrococcus*, *Staphylococcus*, unidentified anaerobes, and yeasts were suggested to be exogenous digestive enzyme-producing organisms (Ray et al., 2012). However, contrary to homoeothermic animals, it is difficult to determine the exact contribution of gut microbiota to the overall digestive enzymes activity in fish (Ray et al., 2012). Nevertheless, several microbial enzymes activities in fish gut were already documented, such as amylase, cellulase, protease, lipase, phytase, tannase, xylanase, and chitinase activities, although activities may change depending on the fish species studied (Bairagi et al., 2002; Ray et al., 2012). In a study where gut bacteria of nine aquaculture freshwater teleost fish were isolated, enumerated, and tested for enzyme activity, almost all bacteria isolates exhibited protease activity (Bairagi et al., 2002). Furthermore, bacteria strains isolated from tilapia (*Oreochromis mossambica*), grass carp (*Ctenopharyngodon idella*), and common carp (*Cyprinus carpio*), showed significant amylase and cellulase activities, and one bacteria strain isolated from silver carp (*Hypophthalmichthys molitrix*) had high lipase activity (Bairagi et al., 2002). The importance of bacteria to the digestive enzymatic production is further supported by the study by Bates et al. (2006), where zebrafish reared in a germ-free environment lacked brush border intestinal alkaline phosphatase activity. Besides production of enzymes, fish gut bacteria also produce vitamins (such as vitamin B_{12}) and PUFA (Ray et al., 2012).

The effects of prebiotics on fish digestive enzymes activities is not much studied, although some authors hypothesised that the observed increase of fish growth could be justified by increased digestive enzymatic activities (Anguiano et al., 2013; Soleimani et al., 2012). Nonetheless, some studies with prebiotics in fish reported increases of proteases, lipases or amylases activities, which are the most measured enzymes (Renjie et al., 2010; Soleimani et al., 2012; Wu et al., 2013; Xu et al., 2009; Zhang et al., 2015). On the other hand, there are also studies that did not detect any significant effect of prebiotics on digestive enzymes activities (Anguiano et al., 2013; Ye et al., 2011). However, studies that simultaneously relate digestive enzymes activities and gut microbiota population are uncommon. Recently, Hoseinifar et al. (2015a) measured both digestive enzymes and gut microbiota community in common carp and observed increased amylase and lipase activities in fish fed 1% scFOS, and increased LAB counts in fish fed 0.5 and 1% scFOS, while protease activity and total bacterial counts were unaltered. The authors related this increased enzymatic activity with a possible increase of exogenous microbial activities, potentially modulated by the prebiotic.

1.2.1.8 Prebiotics dosage

The study of the effects of supplements dosages is a very important approach to determine the level of supplement that provides the best benefits. With dose-response studies it is possible to discard levels of dietary supplementation that do not bring any benefits, and levels that might cause damages or be toxic. Indeed, some works reported adverse effects in fish growth and gut health, when using prebiotics (Cerezuela et al., 2013a; Hoseinifar et al., 2011b; Olsen et al., 2001), which might be connected with the use of inadequate dosages.

Furthermore, it is essential that prebiotics are provided in the diets at an adequate level, which may change depending on fish species, size, prebiotic type, and rearing conditions (Merrifield et al., 2010). Consequently, prebiotic concentration studies are crucial to define the adequate feeding protocol that provides the best results for each species and rearing conditions.

Olsen et al. (2001) and Cerezuela et al. (2013a) reported that inulin induced deleterious effects in the gut of Arctic charr (*Salvelinus alpinus*) and gilthead sea bream, respectively, effects somehow similar to the ones reported in salmon fed SBM. Olsen et al. (2001) fed Arctic charr with diets supplemented with 15% inulin and observed several gut damages, including damages on the enterocytes that seemed connected with the accumulation of lamellar structures, which might be due to inulin absorbed in excess. If that was the case, it means that inulin that could not be degraded by the cells was being accumulated to a point that impaired cell function. Thus, it seems that 15% inulin is clearly above the level that could be supplemented to fish diets. On the other hand, Cerezuela et al. (2013a) fed gilthead sea bream with only 1% inulin and also observed signs of gut oedema and inflammation. These results concur to conclude that correct evaluation of a correct dietary prebiotic supplementation level is necessary to preserve the mucosal integrity of fish gut.

In other studies, authors observed that the beneficial effects perceived with one supplementation level were not visible or even contradict results with other levels (Hoseinifar et al., 2011a; Hoseinifar et al., 2011b; Torrecillas et al., 2007). For instance, in a study with beluga fed oligofructose fish growth was affected by prebiotic level. While feeding fish with 1 and 2% oligofructose had no effect on fish growth, 3% oligofructose resulted in adverse effects on growth performance (Hoseinifar et al., 2011b). This could be related with the inability of intestinal bacteria to ferment the excess of prebiotic provided in the diet. Although not statistically different from the control, diets supplemented with 3% oligofructose produced lower leucocyte count and lymphocyte percentage leading the authors to conclude that this prebiotic level had adverse effects on beluga immunological status (Hoseinifar et al., 2011a). Another example is that of a study with European sea bass fed 0.2 or 0.4% MOS, where the activity of circulating neutrophils were lower than the control in fish fed with 0.2% MOS, while the highest values were recorded in fish fed 0.4% MOS (Torrecillas et al., 2007). Therefore it is evident that the line between beneficial or negative effects of prebiotics may be very thin, and this aspect deserves to be further studied.

1.2.1.9 Rearing conditions

Results obtained when testing a prebiotic may change depending on the animal's rearing conditions. Of all rearing conditions variables, temperature is one of the most important since fish are poikilothermic animals that are constantly subjected to seasonal and daily changes of water temperature. Consequently, temperature modulates fish growth, feed ingestion and utilization, nutrient digestibility, and the activity of key enzymes of intermediary metabolism (Couto et al., 2008, 2012; Enes et al., 2006a, 2008a; Moreira et al., 2008), besides modulating gut microbiota growth (Bucio et al., 2006; Hagi et al., 2004).

As pointed by Ringø et al. (2010), prebiotic supplementation effects may also change depending of the seasonal cycle. Surrounding environment may have greater effects on fish health than the diet, which may confuse interpretation of prebiotic effects. Thus, these are topics that deserve particular attention. As discussed above, the first target of prebiotics are gut bacteria, and it is known that seasonality affects the resident intestinal microbial communities in fish (Bucio et al., 2006; Hagi et al., 2004). For instance, a decrease in total bacteria viable counts and LAB at winter temperatures compared to summer temperatures has been reported (Al-Harbi and Uddin, 2004; Hagi et al., 2004). This increases the importance of studying the influence of rearing temperature on prebiotic effects. However, the effect of temperature on fish bacterial communities is scarcely studied (Al-Harbi and Uddin, 2004; Bucio et al., 2006; Hagi et al., 2004), and the effect of temperature on prebiotics action was never studied (Ringø et al., 2010).

Besides changes in total bacteria numbers, temperature may also change the predominant bacterial type. For instance, in silver carp and in common carp, *Lactococcus lactis* is the predominant LAB in summer temperatures whereas *L. raffinolactis* thrives at winter temperatures (Hagi et al., 2004). Bucio et al. (2006) reported that *Lactobacillus* were more abundant at high temperatures than at low temperatures in perch (*Perca fluviatalis*), carp (*Abramis brama*), and rudd (*Scardinius erithrophthalmus*) inhabiting a river environment. Al-Harbi and Uddin (2004) reported considerable numbers of *Pseudomonas* spp. in hybrid tilapia (*Oreochromis niloticus × O. aureus*) only at winter temperatures. However, it should be noted that these studies were all done with freshwater fish species.

1.2.2 Prebiotics studied in this thesis

1.2.2.1 Short-chain fructooligosaccharides (scFOS)

Inulin, FOS, oligofructose and scFOS are all composed by fructose oligomers, with inulin being used as a generic term covering all ß-(2-1) linear molecules. While inulin usually represents polymers, oligofructose represent oligomers. Oligofructose and FOS are considered synonymous names for mixtures of small inulin oligomers with a degree of polymerization (DP) lower than ten (Roberfroid, 2007).

FOS are prebiotics commonly used in humans and farm animals, and occur naturally in onion, wheat, rye, shallots, tomatoes, bananas, garlic, and Jerusalem artichokes. FOS can also be produced from sucrose or inulin by the action of enzymes with transfructosylating activity isolated from fungi, bacteria, and yeast (Bali et al., 2015). It consists of short and medium chains of ß-D-fructans in which fructosyl units are bound by ß-(2-1) glycosidic linkages attached to a terminal glucose unit (Figure 4) (Ringø et al., 2010). FOS supports growth and survival of bacteria present in the GI tract, such as *Lactobacillus* and *Bifidobacterium*, which possess ß-fructosidase to hydrolyse FOS ß-(2-1) glycosidic bonds (Ringø et al., 2010).

scFOS have the same composition as FOS but with a lower DP (between 1 and 5). Thus, for each glucosyl unit there are 1 to 5 fructosyl units (Figure 4). Onion is the plant with the highest content of scFOS, ranging from 25-40% on a dry matter basis, of which 97% are scFOS with less than 5 fructose units (Bornet et al., 2002).

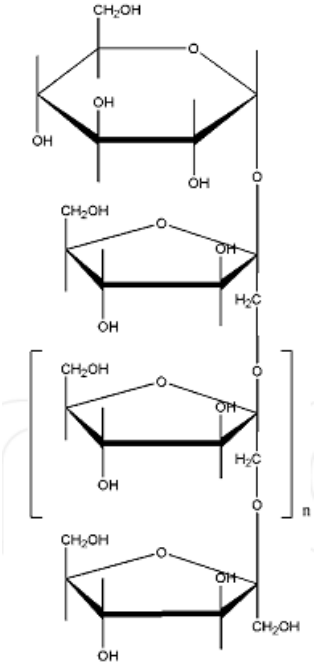

Figure 4 – Structure of fructooligosaccharides (n= 5-10) or short-chain fructooligosaccharides (n= 1-5), a linear fructosyl polymer linked by β-(2,1) bonds, attached to a terminal glucosyl residue by an α-(1,2) bond
(Meyer et al., 2015)

In mammals, FOS were reported to (Bali et al., 2015; Bornet et al., 2002; Gibson and Roberfroid, 1995):

- improve the absorption of various ions, including calcium, magnesium and iron;
- in situ stimulate the growth of certain resident (endogenous/commensal) bacteria and activate bacteria metabolism;
- increase faecal excretion of nitrogen and decrease uraemia;
- regulate glucose and lipid metabolism;
- reduce the incidence of colon tumours and concomitantly develop GALT;
- have an immunomodulatory effect;
- have anti-oxidant properties.

In fish, FOS has been assessed in several fish species (Table 1), while scFOS was only studied to a minor extent in aquatic animals and information is limited to hybrid tilapia (Hui-Yuan et al., 2007; Zhou et al., 2009), Pacific white shrimp (*Litopenaeus vannamei*) (Li et al., 2007; Zhou et al., 2007), rainbow trout (Řehulka et al., 2011) and common carp (Hoseinifar et al., 2015a). Moreover, scFOS was also evaluated in Atlantic salmon (*Salmo salar*) in a symbiosis study with *Pediococcus acidilactici* (Abid et al., 2013).

In most studies, both FOS and scFOS positively improved some of the analysed parameters, such as growth performance, LAB population, and immune parameters. A detailed review of FOS and scFOS studies in fish, including the main results obtained, are present in Table 1. Studies with inulin, or FOS in the form of inulin, are not presented.

Table 1 – Use of fructooligosaccharides (FOS) in fish and short-chain fructooligosaccharides (scFOS) in aquatic animals.

Prebiotic	**Dosage and administration length**	**Fish species and weight (g)**	**Results**	**References**
FOS	0.1% - 17 days	Miiuy croaker (*Miichthys miiuy*) 1.5±0.5g	↗nitrogen digestibility →phosphorous in water and faeces ↘faecal nitrogen	Tian-xing et al. (2005)
	2% - 29-55dph	Turbot 45.5±1.9mg	↗growth, bacterial diversity, growth of *Bacillus* sp. →survival	Mahious et al. (2006)
	2% - 24 and 48 h (*in vitro*)	Red drum Sub-adult	→acetate, propionate, butyrate, total SCFAs, gut microbiota (DGGE)	Burr et al. (2008b)
	1.5 and 3% - 20 days	Sleepy cod (*Oxyeleotris lineolatus*) ≈45g	↗growth, FCR, RBC, Hb, protease, amylase and lipase in stomach and gut	Renjie et al. (2010)
	0.2 and 0.4% - 10 weeks	Large yellow croaker (*Larimichthys crocea*) 7.8±0.7g	→growth, survival, FE, HSI, respiratory burst, lysozyme, ACH50, SOD, survival after a 10 day *Vibiro harveyi* challenge	Ai et al. (2011)
	1, 2 and 3% - 7 weeks	Beluga 18.8±0.8g	↗Ht (2%), lymphocyte % (1 and 2%) →RBC, WBC, Hb, MCH, MCHC, MCV, neutrophil, monocyte, eosinophil, serum ALT, AST, ALP, LDH, glucose, total protein ↘cholesterol (2%)	Hoseinifar et al. (2011a)
	1, 2 and 3% - 7 weeks	Beluga ≈ 19g	↗survival (2%), gut autochthonous LAB log CFU g^{-1} (2%) →growth, FCR, PER, HSI, body composition, gut autochthonous LAB % ↘gut autochthonous TVC (3%)	Hoseinifar et al. (2011b)
	0.5% - 56 days	Japanese flounder ≈21g	→growth, FI, FCR, CF, body composition, lysozyme, phagocytic	Ye et al. (2011)

Prebiotic	Dosage and administration length	Fish species and weight (g)	Results	References
			% and index, cholesterol, HDL-C, protease, amylase ↘triglycerides, LDL-C	
	1, 2 and 3% - 7 weeks	Caspian roach 0.67±0.03g	↗growth, Ig, lysozyme, ACH50 (2 and 3%), survival after salinity stress challenge, amylase (2 and 3%), lipase (2 and 3%), protease ↘FCR (2 and 3%)	Soleimani et al. (2012)
	1 and 2% - 11 weeks	Stellate sturgeon (*Acipenser stellatus*) 30.2±0.1g	↗growth, PER, gut autochthonous TVC, gut autochthonous LAB (1%), lysozyme (1%), WBC →respiratory burst, differential WBC, Ht, Hb ↘FCR	Akrami et al. (2013)
	0.5 and 1% - 49 days	Rainbow trout ≈150g	↗growth, body gross energy, body Ca (1%) →FI, FCR, distal gut *Aeromonas* spp. *Pseudomonas* spp. *Vibrio* spp. *Flavobacterium* spp. Gram-positive bacteria, digesta pH ↘fillet protein	Ortiz et al. (2013)
	0.05, 0.1, 0.2, 0.4 and 0.8% - 8 weeks	Blunt snout bream 1.42±0.01g	↗growth, survival (0.1, 0.2, 0.4 and 0.8%), amylase (0.4 and 0.8%), protease, body lipid (0.2 and 0.4%), microvilli length →lipase, body protein and ash ↘FCR (0.4 and 0.8%), body moisture (0.4%)	Wu et al. (2013)
	0.3 and 0.6% - 8 weeks	Triangular bream (*Megalobrama terminalis*) 30.5±0.5g	↗WBC, AKP, globulin (0.6%), ACH50, PO, IgM, liver and plasma SOD →RBC, ACP, lysozyme, serum protein, liver and plasma CAT and GPX, survival after *Aeromonas hydrophila* challenge ↘liver MDA (0.6%), plasma MDA	Zhang et al. (2013)
	1, 2 and 3% - 7 weeks	Common carp 3.23±0.14g	↗survival (3%), WBC (3%), respiratory burst, gut autochthonous TVC, gut autochthonous LAB (2 and 3%), survival after salinity stress challenge →growth, CF, FCR, differential WBC, Hb, Ht	Hoseinifar et al. (2014b)
	0.4 and 0.8% - 8 weeks	Blunt snout bream 13.8±0.04g	↗plasma total protein, IgM, ACP, ACH50, nitrogen monoxide, liver SOD and CAT, resistance to high	Zhang et al. (2014)

Prebiotic	Dosage and administration length	Fish species and weight (g)	Results	References
			heat stress (all with level 0.4%), lysozyme, HSP70 expression →liver MDA, HSP90 expression, plasma lactate ↘plasma cortisol (04%) and glucose (0.4%)	
	0.3 and 0.6% - 8 weeks	Triangular bream 30.5±0.5g	↗growth (0.6%), survival, body lipid (0.6%), protease, lipase (0.6%), Na^+, K^+-ATPase, mid gut microvilli length →CF, body moisture, ash and protein, amylase ↘FCR, HSI, VSI	Zhang et al. (2015)
scFOS	0.08 and 0.12% - 8 weeks	Hybrid tilapia 5.55±0.02g	↗growth (0.12%)→FI, FCR, HSI, CF, survival, gut autochthonous counts of *Vibrio parahaemolyticus, A. hydrophila, Lactobacillus* sp., and *Streptococcus faecalis*	Hui-Yuan et al. (2007)
	0.1% - 8 weeks	Hybrid tilapia 1.24±0.01g	Gut autochthonous bacterial community different from control group, presence of uncultured bacterium clones and *Thiothrix eikelboomii*	Zhou et al. (2009)
	0.025, 0.05, 0.075, 0.1, 0.2, 0.4 and 0.8% - 6 weeks	Pacific white shrimp 75.4±0.8g	↗haemocyte respiratory burst (0.1 and 08%), support growth of certain bacterial species →growth, FE, survival, total haemocyte count, haemocyte phenoloxidase	Li et al. (2007)
	0.04, 0.08, 0.12 and 0.16% - 8 weeks	Pacific white shrimp ≈0.17g	↗growth, scFOS affected gut microbiota →FI, survival ↘FCR	Zhou et al. (2007)
	0.1% - 105 days	Rainbow trout ≈240g	→growth, FCR, survival ↘serum creatinine, Na^+, alkaline phosphatase	Řehulka et al. (2011)
	0.5 and 1% - 7 weeks	Common carp 550±20mg	↗survival (1%), body lipid, amylase (1%), lipase (1%), gut autochthonous LAB →growth, CF, FCR, body moisture and ash, protease, gut autochthonous TVC ↘body protein	Hoseinifar et al. (2015a)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. ACH50: alternative complement activity; ACP: acid phosphatase; AKP: alkaline phosphatase; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CAT: catalase; CF: condition factor; CFU: colony forming units; DGGE: denaturing gradient gel electrophoresis; dph: days post hatching; FCR: feed conversion ratio; FE: feed efficiency; FI: feed intake; Hb:

haemoglobin; HDL-C: high-density lipoprotein cholesterol; HSI: hepatosomatic index; Ht: haematocrit; Ig: immunoglobulin; IgM: immunoglobulin M; LAB: lactic acid bacteria; LDH: lactate dehydrogenase; LDL: low-density lipoprotein cholesterol; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; MDA: malondialdehyde content; PER: protein efficiency ratio; PO: phenoloxidase; RBC: red blood cells; SCFAs: short-chain fatty acids; SOD: superoxide dismutase; TVC: bacteria total viable counts; VSI: visceral index; WBC: white blood cells.

1.2.2.2 Xylooligosaccharides (XOS)

XOS is an emerging prebiotic, gaining importance as functional ingredient in pharmaceutics, feed, and food formulation (Aachary and Prapulla, 2011). XOS are sugar oligomers made up of xylose units, and appears naturally in bamboo shoots, fruits, vegetables, milk, and honey. Industrially, XOS is produced by chemical or enzymatic hydrolysis of xylan, which is the major component of lignocellulosic raw materials (Vázquez et al., 2000). The chemical structure of XOS depends on the xylan source; thus, structures can vary in DP, monomeric units, and type of linkages. XOS generally consists of chains of xylose linked by β-(1-4) bonds, with a DP ranging from 2 to 10, which are known as xylobiose (DP=2), xylotriose (DP=3), etc. (Figure 5) (Aachary and Prapulla, 2011). Xylobiose is considered a xylooligosaccharide, even though for other purposes the concept “oligo” is associated with higher DP (Vázquez et al., 2000).

XOS supports growth and survival of bacteria present in the GI tract, such as *Bifidobacterium*. An efficient and complete degradation of XOS requires the cooperation of different enzymes including β-xylosidase, α-glucuronidase, α-larabinosidase, or acetyl xylan esterase (Aachary and Prapulla, 2011).

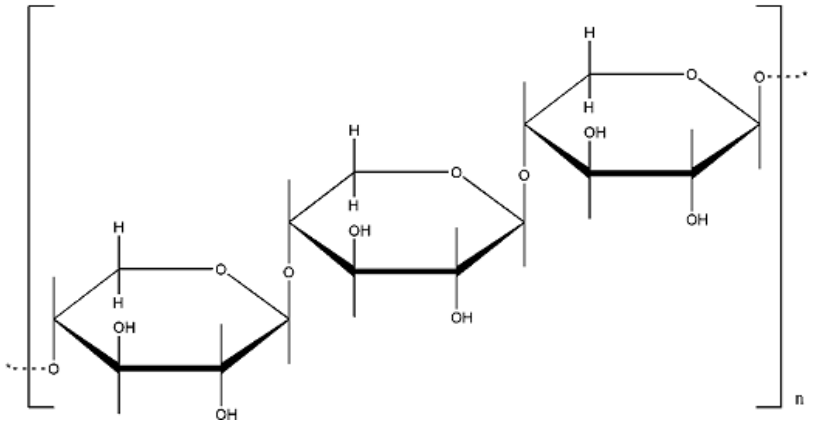

Figure 5 – Partial structure of xylooligosaccharides (n=3-6) produced by enzymatic hydrolysis of xylan hemicelluloses, catalysed by β-xylanases.
(Meyer et al., 2015)

In mammals, XOS were reported as having (Aachary and Prapulla, 2011; Broekaert et al., 2011):

- immunomodulatory effect;
- anti-cancerous activity in the colon;
- anti-microbial activity;
- anti-oxidant activity;

- effects on stool frequency and consistency;
- anti-infection and anti-inflammatory properties;
- growth promoting effects on beneficial bacteria;
- improve mineral uptake effects;
- glucose and lipid metabolism regulating effects.

In fish, XOS was assessed only in allogynogenetic crucian carp (Xu et al., 2009), turbot (Li et al., 2008), Caspian white fish (*Rutilus frisii* kutum) (Hoseinifar et al., 2014a), and European sea bass (Abdelmalek et al., 2015). The main beneficial effects reported for XOS in fish were improvement of growth and feed utilization, stimulation of immune parameters, and increased levels of gut LAB. A more detailed review of XOS studies in fish, and the main results observed, is presented in Table 2.

Table 2 – Use of xylooligosaccharides (XOS) in fish.

Prebiotic	**Dosage and administration length**	**Fish species and weight (g)**	**Results**	**References**
XOS	0.04% - 72 days	Turbot 151.3±11.3g	↗growth, complement C3 and C4, phagocytes (%), lysozyme →FI, serum SOD ↘FCR	Li et al. (2008)
	0.005, 0.01 and 0.02% - 45 days	Allogynogenetic crucian carp ≈17g	↗growth, intestinal protease (0.01%) and amylase, hepatopancreatic protease (0.005 and 0.01%) and amylase (0.01%)	Xu et al. (2009)
	1, 2 and 3% - 8 weeks	Caspian white fish 1.54±0.03g	↗skin mucus total protein (3%), gut TVC, gut autochthonous LAB (2 and 3%), bactericidal activity of skin mucus against *Streptococcus faecium* (2 and 3%), *Serratia marcescens* (3%), *Staphylococcus aureus* (3%), and *Escherichia coli* (3%) →growth, CF, FCR, morphology of distal gut	Hoseinifar et al. (2014a)
	0.5 and 1% - 12 weeks	European sea bass 4.75±0.69g	↗growth, FE (0.5%), PER (0.5%), survival after challenge with *A. hydrophila* (1%), RBC pre and pos-C, WBC pre-C, Hb pre and pos-C, Ig pre-C, Ig pos-C (0.5%), lysozyme pre and pos-C →liver weight, HSI, liver morphology ↘WBC pos-C, serum protein pre-C (1%)	Abdelmalek et al. (2015)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. CF: condition factor; FE: feed efficiency; FCR: feed conversion ratio; FI: feed intake; Hb: haemoglobin; HSI: hepatosomatic index; Ig: immunoglobulin; LAB: lactic acid bacteria;

PER: protein efficiency ratio; pos-C: pos-challenge; pre-C: pre-challenge; RBC: red blood cells; SOD: superoxide dismutase; TVC: total viable counts; WBC: white blood cells.

1.2.2.3 Galactooligosaccharides (GOS)

GOS are also known as oligogalactosyllactose, oligogalactose, oligolactose, or TOS (Sangwan et al., 2011). GOS are defined as mixtures of substances produced from lactose, with a DP between 2 and 10, where one of the units is a terminal glucose and the remaining saccharides units are galactose (Figure 6) (Macfarlane et al., 2008; Torres et al., 2010). β-galactosidases, which have transgalactosylation activities and are produced by fungi, yeasts, and bacteria, are used to enzymatically process lactose and produce several GOS oligomers of different chain lengths. Although GOS can be produced from cows milk lactose, the main raw material used for its production for commercial products is whey-derived lactose (Macfarlane et al., 2008). Other possible source of GOS are soybeans (Sangwan et al., 2011).

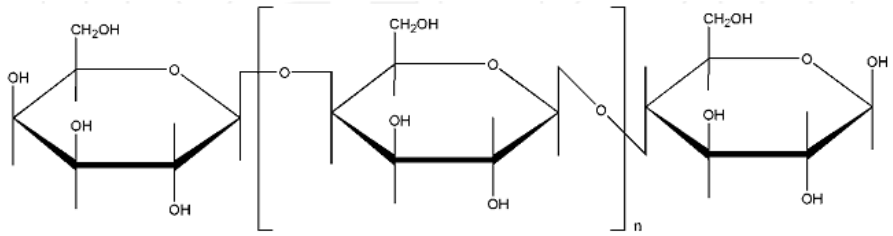

Figure 6 – Structure of a galactooligosaccharide derived from lactose, a β-(1,4) linked galactosyl oligomer (n=1-4), attached to a terminal glucosyl residue by a β-(1,4) bond.
(Meyer et al., 2015)

In mammals, GOS were reported as (Macfarlane et al., 2008; Sangwan et al., 2011; Torres et al., 2010):

- promoting the growth of beneficial bacteria, namely *Bifidobacterium* and *Lactobacillus*;
- having antipathogenic activity;
- protecting against enteric infections;
- increasing mineral absorption;
- being immunomodulators for the prevention of allergies and gut inflammatory conditions;
- having trophic effects of SCFAs on the colonic epithelium;
- controlling serum lipid and cholesterol levels;
- affecting faecal bulking;
- reducing toxigenic microbial metabolism that may reduce risk factors for colon cancer.

In fish, GOS was only assessed in red drum (Burr et al., 2008a; Zhou et al., 2010), hybrid striped bass (Burr et al., 2010), Atlantic salmon (Grisdale-Helland et al., 2008), Caspian roach (Hoseinifar et al., 2013), rainbow trout (Hoseinifar et al., 2015c) and goldfish (*Carassius auratus*) (Raggi and Gatlin III, 2012). There is also a report on TOS use in red drum (Buentello et al., 2010). The main positive results reported of using GOS in fish are: improved growth and

feed utilization, improved feed digestibility, higher survival after bacterial or salinity challenges, and stimulated immunity. See Table 3 for a more detailed review of the species where GOS and TOS were studied in fish and the main results observed.

Table 3 – Use of galactooligosaccharides (GOS) and transgalactooligosaccharides (TOS) in fish.

Prebiotic	**Dosage and administration length**	**Fish species and weight (g)**	**Results**	**References**
GOS	1% - 3 weeks	Red drum ≈500g	↗protein, organic matter, energy and carbohydrates digestibility in SBM based diet ↘lipid digestibility	Burr et al. (2008a)
	1% - 8 weeks	Red drum 7.1±0.1g	↗growth, lysozyme, pyloric caeca, proximal and mid gut microvillus height →survival, FE, PER, HSI, CF, NBT, proximal, mid and distal gut fold height and total enterocyte height, pyloric caeca total enterocyte height	Zhou et al. (2010)
	1% - 4 months	Atlantic salmon 200.2±0.6g	↗energy digestibility, nitrogen non-faecal loss, energy non-faecal nitrogen →growth, FI, FE, body moisture, lipid, ash and energy, NBT, lysozyme, lipid and protein digestibility ↘body protein, nitrogen retained	Grisdale-Helland et al. (2008)
	0.5% (*in vitro*)	Hybrid striped bass ≈200g	↗butyrate (48h) →acetate and propionate (48h), DGGE, presence of *Fusobacteria bacterium*	Burr et al. (2010)
	1% - 8 weeks	Hybrid striped bass 344.4±11g	→growth, FE, PER, body composition different type of microbial community in the gut	Burr et al. (2010)
	1% - 8 weeks	Goldfish ≈15g	→protein, organic matter and carbohydrates digestibility in SMB based diet, DGGE ↘lipid digestibility	Raggi and Gatlin III (2012)
	1 and 2% - 7 weeks	Caspian roach 1.36±0.03g	↗growth (2%), survival, survival after a salinity stress challenge (2%), gut autochthonous LAB →CF, body composition, gut autochthonous TVC ↘FCR (2%)	Hoseinifar et al. (2013)
	1% - 8 weeks	Rainbow trout 15.0±0.5g	↗lysozyme, ACH50, respiratory burst activity, skin mucus protein, survival after *Streptococcus iniae* challenge, bactericidal activity skin mucus against *S. faecium*, *S. iniae*, *Serratia marcescens* and *Escherichia coli*	Hoseinifar et al. (2015c)

Prebiotic	Dosage and administration length	Fish species and weight (g)	Results	References
TOS	1% - 4 + 2 weeks	Red drum 10.9±0.2g	↗lysozyme, IC-SOAP →growth, survival after *Amyloodinium ocellatum* challenge, FE, body composition, EX-SOAP, NBT	Buentello et al. (2010)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. ACH50: alternative complement activity; CF: condition factor; DGGE: denaturing gradient gel electrophoresis; EX-SOAP: extracellular superoxide anion production; FCR: feed conversion ratio; FE: feed efficiency; HSI: hepatosomatic index; IC-SOAP: intracellular superoxide anion production; LAB: lactic acid bacteria; NBT: blood neutrophil oxidative radical production; PER: protein efficiency ratio; SMB: soybean meal; TVC: total viable counts.

1.3 Fish species studied in this thesis

1.3.1 Turbot (*Scophthalmus maximus*, Rafinesque, 1810)

Turbot (Figure 7) is a marine demersal flatfish of the Scophthalmidae family. As a benthic species, turbot lives on sandy and muddy bottoms, at depths from 20 to 70m and can live at temperatures between 5-25°C. It is relatively abundant in Europe, from 68°N down to Morocco 30°N, and the Mediterranean Sea. Spawning occurs between February and April in the Mediterranean, and between May and July in the Atlantic (FAO, 2005b; Person-Le Ruyet et al., 1991).

Turbot aquaculture was initiated in the early 1970s in Scotland. Soon it was also introduced in France and Spain due to its high commercial value and high growth rate in intensive rearing. Nowadays, the main turbot producer is Spain, but turbot is also produced in Denmark, Germany, Iceland, Ireland, Italy, Norway, Wales (UK), Portugal, Chile, and China (FAO, 2005b; Person-Le Ruyet et al., 1991). Production has oscillated through the years, with a total production of 76 998 tonnes (representing $ 637 995 000) in 2013, of which 2 453 tonnes ($ 16 907 000) were produced in Portugal (FIGIS, 2015). On-growing production costs are higher in tanks than in cages, but the rearing method most used is in tanks, since off-shore rearing is still in an experimental stage (FAO, 2005b).

Some rearing problems still persist associated with disease prevention and control of ectoparasites (*Neoparamoeba pemaquidensis, Trichodina* spp., *Philasteridis dicentrarchi*), endoparasites (*Tetramicra brevifilum, Enteromysum scophthalmi*), and bacterium (*Tenacibaculum maritimun, Aeromonas salmonicida, Streptococcus parauberis, Vibrio anguillarum*) (FAO, 2005b).

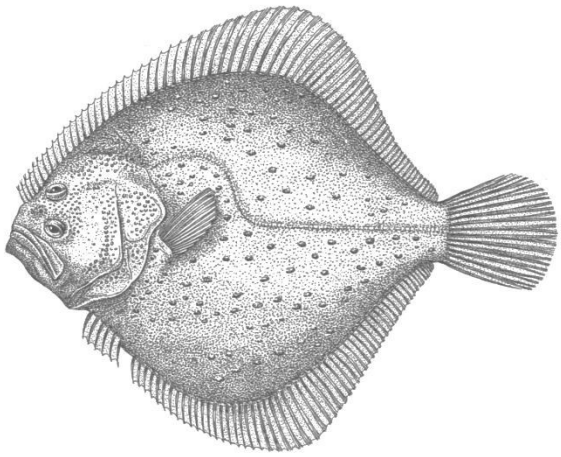

Figure 7 – Turbot (*Scophthalmus maximus*)
(WoRMS, 2015)

1.3.1.1 Nutritional recommendations

Turbot is a carnivorous fish, with juveniles usually feeding on molluscs and crustaceans and adults mainly feeding on fish and cephalopods (FAO, 2005b).

Table 4 presents a summary of the dietary macronutrient recommendations for turbot.

Regarding protein requirements, first studies reported a low requirement level, results showing that fish fed a diet with 35% protein had better protein utilization and similar growth to fish fed a diet with 50% protein (Adron et al., 1976). Thereafter, Caceres-Martinez et al. (1984) using semi-purified diets reported a dietary protein requirement of about 70% for best growth and feed conversion, and Devesa (1994) recommended a dietary protein level of 45-50% for maximum growth. A more recent study reported that about 50% protein provided optimal growth of turbot juveniles (Lee et al., 2003).

Fish require the same essential amino acids (EAA) as most farm animals, except arginine, that is essential for fish and birds, but not for mammals (Kaushik, 1998). Thus, fish have requirements for arginine, phenylalanine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine. Despite the importance of turbot in aquaculture, EAA requirements for this species were only estimated by the ideal protein method, based on lysine requirements and whole-body EAA composition of turbot (Kaushik, 1998). Since no data for lysine requirement was available at that time, the author used a requirement value of 5.0g/16g nitrogen (N), based on known requirements for other fish. Requirements, presented as g/16g N, were estimated to be 4.8g for arginine, 5.3g for phenylalanine + tyrosine, 1.5g for histidine, 2.6g for isoleucine, 4.6g for leucine, 2.7g for methionine + cysteine, 2.9g for threonine, 0.6g for tryptophan, and 2.9g for valine (Kaushik, 1998). More recently, Peres and Oliva-Teles (2008) based on a dose-response study estimated lysine requirement for turbot juveniles to be 5.0g/16g N. Based on this lysine requirement value and the A/E (specific EAA content × 1000/total EAA) ratio determined from whole body amino acid profile, estimated

the EAA requirements of turbot juveniles, expressed in g/16g N, to be 4.22g for arginine, 2.54g for phenylalanine, 1.90g for tyrosine, 1.28g for histidine, 2.59g for isoleucine, 4.47g for leucine, 1.68g for methionine, 2.37g for threonine, and 2.74g for valine.

Early studies reported that with a high protein diet (70%), a dietary level of 10% lipid produced the best growth and feed conversion of turbot juveniles, and that 15 or 20% of dietary lipids negatively affected growth and feed conversion (Caceres-Martinez et al., 1984). However, with diets including less than 70% protein, lipids did not produce such negative effects (Caceres-Martinez et al., 1984). In a study with marketable size turbot, 10 or 15% lipids promoted the best growth performance, while higher levels, 20 and 25%, led to decreased growth and changes in body composition (Regost et al., 2001). Recently, Sevgili et al. (2014) estimated 13% lipids as the optimum dietary lipid level for turbot juveniles, since higher levels reduced growth and feed utilization, had no protein sparing effect, and resulted in higher carbon losses.

The highly unsaturated fatty acids of the n-3 series (n-3 HUFA), namely eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are considered essential for marine fish, as they are not able to synthetize them from the n-3 precursor alpha-linolenic acid (ALA, 18:3n-3) at least at a sufficient rate to meet requirements (Kanazawa, 1985). Gatesoupe et al. (1977b) estimated the EFA requirements of turbot to be 0.8% of EPA + DHA. In another experiment, the same authors demonstrated that 2.7% ALA could be used instead of the 0.8% n-3 HUFA (Gatesoupe et al., 1977a). Later, Castell et al. (1994) showed that 1% arachidonic acid (ARA, 20:4n-6) is also essential for turbot juveniles.

Studies about carbohydrates utilization by turbot are scarce. Turbot seems to tolerate up to 20% of cooked maize starch, while raw starch is not so well utilized as cooked starch (Jollivet et al., 1988). On the other hand, Caceres-Martinez et al. (1984) obtained the best growth and FE of turbot juveniles with only 2.7% carbohydrate.

The only vitamins requirements studied in turbot were thiamine, pyridoxine, vitamin C, and vitamin E. Vitamin requirements were reported to be 0.6 to 2.6mgKg^{-1} of thiamine (Cowey et al., 1975), 1.0 to 2.5 mgKg^{-1} of pyridoxine (Adron et al., 1978), 50-100mgKg^{-1} of vitamin C (Devesa, 1994) and 30mgKg^{-1} of vitamin E (Devesa, 1994).

Mineral requirements for turbot were not yet established, thus mineral requirements levels of other marine fish are used in turbot diets.

Table 4 – Summary of the main nutritional groups (protein, lipids and carbohydrates) recommendations for turbot.

Feeding behaviour	Nutrient	Fish weight (g)	Recommendation level	References
Carnivorous	Protein	≈11g	35%	Adron et al. (1976)
		≈10g	70%	Caceres-Martinez et al. (1984)
		-	45-50%	Devesa (1994)
		89g	50%	Lee et al. (2003)
	Lipids	≈10g	10% if protein =70% or 15-20% if protein <70%	Caceres-Martinez et al. (1984)
		657±6g	10-15%	Regost et al. (2001)
		54.4±0.2g	13%	Sevgili et al. (2014)
	Carbohydrates	≈10g	2.7% carbohydrates	Caceres-Martinez et al. (1984)
		150-550g	Up to 20% of cooked maize starch	Jollivet et al. (1988)

1.3.1.2 Fish meal replacement by plant feedstuffs

Turbot seems to tolerate high levels of PF in the diets. Fournier et al. (2004) reported that FM can be reduced to only 20% without negative effects on growth performance, in diets with lupin, corn gluten, and wheat gluten meal, supplemented with crystalline amino acids, while diets with only 10% FM reduced growth. Bonaldo et al. (2011) concluded that a mixture of SBM, wheat gluten meal, and corn gluten meal, can replace up to 52% FM in the diets without reducing feed intake (FI) and with no need of amino acids supplementation. However, for optimal growth and nutrient utilization, a FM substitution of only 39% FM is recommended. Independently of the FM replacement level (25, 39, 52, and 66%) no deleterious effects were observed on fish's intestinal histology.

1.3.1.3 Prebiotics use

Despite the many studies about the use of probiotics in turbot, prebiotics application have been understudied in this species (Dimitroglou et al., 2011a). Mahious et al. (2006) studied the effect of 2% inulin, oligofructose, and lactosucrose as prebiotics during turbot weaning. Oligofructose improved fish growth and was also the only prebiotic that promote the growth of *Bacillus* spp., which represented 14% of total bacteria isolates in that group. These results allowed the authors to conclude that *Bacillus* spp. might use oligofructose as a single carbon source, and this justified the positive effect of this prebiotic on fish growth.

In other study, the effect of XOS (0.04%) was evaluated on nonspecific immunity and growth of turbot juveniles (Li et al., 2008). Dietary supplementation with XOS led to a significant increase in fish growth and enhanced nonspecific immunity, mainly due to an increase in complement C4 activity and in phagocytes percentage.

Chitosan (COS) was also reported as having potential as prebiotic, since it stimulates the growth of some beneficial enteric bacteria (Lee et al., 2002). Thus, Cui et al. (2013) studied the effect of supplementing turbot diets with graded levels (0.0075, 0.015, 0.03, 0.06, and 0.12%) of COS with rare earth (COS-REE). Rare earth elements are used in China as feed additives in animal production, and include lanthanoids in group III of the periodic table, scandiumare, and yttrium. COS-REE supplemented to diets enhanced turbot growth, innate immunity, and disease resistance, being 0.03% the dietary recommend level (Cui et al., 2013).

A review of the prebiotics already tested in turbot and the main results observed is presented in Table 5.

Table 5 – Prebiotics use in turbot.

Prebiotic	**Fish weight (g)**	**Dosage and administration length**	**Results**	**References**
Inulin, oligofructose and lactosucrose	45.5±1.9mg	2% - 29-55dph	↗growth, bacterial diversity, growth of *Bacillus* sp. (oligofructose) →survival	Mahious et al. (2006)
XOS	151.3±11.3g	0.04% - 72 days	↗growth, complement C3 and C4, phagocytes (%), lysozyme →FI, serum SOD ↘FCR	Li et al. (2008)
COS-REE	12.1 ± 0.1g	0.0075, 0.015, 0.03, 0.06 and 0.12% - 8 weeks	↗growth (0.03 and 0.06%), phagocytic index (0.015, 0.03, 0.06 and 0.12%) →survival, body composition, serum SOD and MDA, survival after *Edwardsiella tarda* challenge ↘FCR, hepatic MT (0.015 and 0.06%)	Cui et al. (2013)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. COS-REE: chitosan oligosaccharide complex with rare earth; dph: days post hatching; FCR: feed conversion ratio; FI: feed intake; MDA: malondialdehyde; MT: metallothionein; SOD: superoxide dismutase.

1.3.2 Gilthead sea bream (*Sparus aurata*, Linnaeus, 1758)

Gilthead sea bream (Figure 8) is an eurythermic (5-32°C) and euryhaline fish belonging to the Sparidae family (Barnabé, 1989; FAO, 2005a). It is a coastal benthopelagic species that lives in

seagrass beds, rocky, and sandy bottoms, as well as in shallow waters at depths of about 30m, although adults may be found at depths of 150m. It is relatively common in the Mediterranean Sea, present along the Eastern Atlantic coasts from Great Britain to Senegal, and rare in the Black Sea. Spawning occurs between October to December, and fish do no spawn in the Black Sea. Young fish normally migrate in early spring to protected coastal waters, and in late autumn fish return to open sea where breeding occur (FAO, 2005a).

Before intensive rearing was achieved in 1988-1989 in Spain, Italy, and Greece, gilthead sea bream were reared extensively in coastal lagoons and in saltwater pond. Nowadays, Portugal is among the main producers, which comprise mostly European and North Africa countries (FAO, 2005a). The production has oscillated through the years, with a total production of 173 062 tonnes (representing $ 1 065 027 000) in 2013, with 810 tonnes ($ 5 840 000) of gilthead sea bream being produced in Portugal (FIGIS, 2015).

The major disease problems affecting gilthead sea bream are related with bacterium (*Photobacterium damselae* subsp. *piscicida* and subsp. *damselae, Vibrio alginolyticus, V. anguillarum, Pseudomonas anguilliseptica*), virus (*Iridoviridae*, Aquareovirus, Virus-like particle) and endoparasites (*Myxidium leei*) (FAO, 2005a).

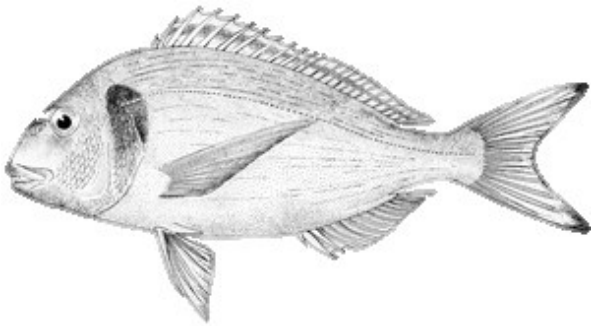

Figure 8 – Gilthead sea bream (*Sparus aurata*)
(FAO, 2005a)

1.3.2.1 Nutritional recommendations

Gilthead sea bream is mainly carnivorous, and feeds mainly on molluscs, crustaceans, and fish, but accessorily it can be herbivorous (FAO, 2005a).

There is some information available regarding the nutritional requirements of this species, however still with some discordant data.

Table 6 presents a summary of the dietary macronutrient recommendations for gilthead sea bream. The first studies about protein requirements were done using semi-purified diets and reported a protein requirement of 40% for maximum growth (Sabaut and Luquet, 1973). Afterwards, other studies reported higher protein requirements, 45-46% for juveniles (Santinha et al., 1996; Vergara et al., 1996b) and 55% protein for fry (Vergara et al., 1996a).

Based on dose-response studies, the following EAA requirements were estimated (in g/16g N): 2.6g for arginine (Luquet and Sabaut, 1974), 5.0g for lysine (Luquet and Sabaut, 1974; Marcouli et al., 2006), 4.0g for methionine + cysteine (Luquet and Sabaut, 1974) and 0.6g for tryptophan (Luquet and Sabaut, 1974). Kaushik (1998) estimated the EAA requirements of gilthead sea bream by the ideal protein method and indicated requirements (in g/16g N) of 5.4g for arginine, 2.9g for phenylalanine + tyrosine, 1.7g for histidine, 2.6g for isoleucine, 4.5g for leucine, 2.4g for methionine + cysteine, 2.8g for threonine, 0.6g for tryptophan and 3.0g for valine. More recently, Peres and Oliva-Teles (2009) used the amino acid deletion method to estimate the ideal protein and based on that and the lysine requirement, reported requirements for the other EAA (in g/16g N) of: 5.55g for arginine, 5.76g for phenylalanine + tyrosine, 1.89g for histidine, 2.55g for isoleucine, 4.75g for leucine, 5.13g for lysine, 2.60g for methionine, 2.98g for threonine, 0.75g for tryptophan, and 3.21g for valine.

Optimum dietary lipid levels for gilthead sea bream juveniles were at first estimated to be 15-16% (Vergara and Jauncey, 1993; Vergara et al., 1996b) but later studies reported values of 21-22% (Santinha et al., 1999; Vergara et al., 1999). Data on HUFA requirements is scarce. Kalogeropoulos et al. (1992) in a study with 1g fish, using diets with 12% lipids from soybean oil (6%) and cod-liver oil (6%), estimated the minimum requirement for EPA and DHA to be 0.9% of the diet. However, HUFA requirements can vary with fish size; for 43g fish HUFA requirement is about 1.9% n-3 HUFA (Ibeas et al., 1994), while for 12g fish a requirement of 1% n-3 HUFA was reported (Ibeas et al., 1996).

Fish do not have specific carbohydrate requirements, but studies indicate that a dietary inclusion of up to 20% native, waxy, or gelatinized maize starch, do not affect fish performance (Couto et al., 2008; Enes et al., 2008b). Moreover, Venou et al. (2003) fed gilthead sea bream with 40% of raw or extruded starch from maize and wheat, and observed that wheat starch lead to a better performance than maize starch.

In a study by Morris et al. (1995), vitamins from the B complex: thiamine, riboflavin, pyridoxine, niacin, and pantothenic acid were proven to be essential by removing each vitamin individually from a B vitamin complex ($mgKg^{-1}$: 69.9 thiamine, 208.3 riboflavin, 48.6 pyridoxine, 800.0 niacin, 305.3 pantothenic acid). Other studies reported requirements between 63.0 and 83.0$mgKg^{-1}$ of nicotinic acid (Morris and Davies, 1995a), 10.0$mgKg^{-1}$ of thiamine (Morris and Davies, 1995c), and 1.97$mgKg^{-1}$ of pyridoxine (Kissil et al., 1981). Later, Morris and Davies (1995b) studying the pyridoxine requirements, did not found any differences in growth of fish fed 0.5, 5.0 or 100.0$mgKg^{-1}$ pyridoxine. Diet supplementation with vitamin C did not promote growth; however, higher protein efficiency ratio (PER) was recorded in fish fed 200.0$mgKg^{-1}$ of vitamin C (Henrique et al., 1998).

The only mineral requirement determined for gilthead sea bream was phosphorus, with a dietary requirement of 0.75% (Pimentel-Rodrigues and Oliva-Teles, 2001).

Table 6 – Summary of the main nutritional groups (protein, lipids and carbohydrates) recommendations for gilthead sea bream.

Feeding behaviour	Nutrient	Fish weight (g)	Recommendation level	References
Carnivorous or accessorily herbivorous	Protein	≈2.6g	40%	Sabaut and Luquet (1973)
		9.8±0.3g	45%	Santinha et al. (1996)
		≈5.3g	46%	Vergara et al. (1996b)
		≈0.8g	55%	Vergara et al. (1996a)
	Lipids	42-46g	16%	Vergara and Jauncey (1993)
		≈5.3g	15%	Vergara et al. (1996b)
		42.5±0.2g	21%	Santinha et al. (1999)
		68-73g	22%	Vergara et al. (1999)
	Carbohydrates	≈13g	40% raw or extruded wheat starch	Venou et al. (2003)
		≈30g	Up to 20% gelatinized maize starch	Couto et al. (2008)
		20g	Up to 20% native or waxy maize starch	Enes et al. (2008b)

1.3.2.2 Fish meal replacement by plant feedstuffs

Gilthead sea bream of 100g fed diets with 75% of FM protein replaced by PF protein (corn gluten meal, wheat gluten, extruded peas, rapeseed meal) and supplemented with crystalline amino acids, grew as well as fish fed a FM-based diet, and had lower FI and higher FE. Only minor effects on fish quality traits, such as higher levels of n-3 polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids in fish fed FM diet, and higher level of n-6 PUFA in fish fed PF diet were reported (De Francesco et al., 2007). In a study with 180g fish, substitution of 60% FM by PF (SBM, soy protein concentrate, peas concentrate, wheat gluten, wheat meal, wheat dried distillers grains with solubles, corn gluten) with supplementation of lysine, arginine, and methionine also did not affect growth performance (Dias et al., 2009). However, lower energy digestibility and lower PER were observed in fish fed the PF diet. Since the control diet was a commercial sea bream diet, made with both FM and PF, the substitution

of 60% FM corresponded to a level of only 13% of marine-derived ingredients accounting as protein sources. In other study with 40g fish, a substitution of 100% of FM by a mixture of PF (wheat gluten, soy protein concentrate, corn gluten meal, and wheat meal) supplemented with methionine, lysine, threonine, and arginine was accomplished producing higher weight gain and lower FCR than fish fed a 100% FM-based diet (Kissil and Lupatsch, 2004). The only disadvantage with that all-PF diet was fish production cost, which was higher than with the FM diet. Gómez-Requeni et al. (2004) in a study with 16g fish, reported that substitution of only 50% FM by PF (corn gluten, wheat gluten, extruded peas, rapeseed meal, sweet white lupin) balanced with indispensable amino acids, reduced fish growth. Overall, this indicates that tolerance of gilthead sea bream to PF may change with fish weight. In fact, in a study with 12.5g juveniles and 112g on-growing fish fed purified antinutrients, it was proved that smaller fish were more sensitive to damages caused by antinutrients (Couto et al., 2014a, b).

1.3.2.3 Prebiotics use

Up to now, only two prebiotics were studied in gilthead sea bream, MOS and inulin (Cerezuela et al., 2008; Cerezuela et al., 2012; Cerezuela et al., 2013a; Cerezuela et al., 2013b; Dimitroglou et al., 2010b; Gültepe et al., 2011; Gültepe et al., 2012; Gültepe et al., 2015).

MOS (from Bio-MOS®) was evaluated in gilthead sea bream at two dietary levels, 0.2 and 0.4% (Gültepe et al., 2011; Gültepe et al., 2012), or only at one level 0.2% (Gültepe et al., 2015). Fish fed both levels of MOS had better growth, FCR, and feed digestibility (Gültepe et al., 2011). On the other side, no effects on fish health, haematology, liver and muscle histopathology were observed (Gültepe et al., 2012; Gültepe et al., 2015). Although MOS had no effect on general fish health, growth and FE improvements led the authors to recommend the inclusion of only 0.2% Bio-MOS® to the diets.

Dimitroglou et al. (2010b) studied the effect of 0.2% and 0.4% MOS on FM-based diets, and of 0.4% MOS on SBM-based diets. In both cases MOS had no effect on fish growth or FE. MOS increased the absorptive surface of the posterior gut of fish fed FM-based diets with 0.4% MOS, while microvilli density and length of the anterior and posterior gut were increased in both FM and SBM groups. MOS effect on GI microbiota was more pronounced in fish fed FM diets, which was reflected by increased species richness and diversity, while in fish fed SBM-based diets no differences in GI microbiota were observed. Authors concluded that dietary SBM exerted a greater effect on gut microbiota than dietary MOS.

The first report on the effect of inulin in gilthead sea bream was related to its effect on the innate immune response, both *in vitro* and *in vivo* (Cerezuela et al., 2008). In the *in vitro* study, no effects were observed on the main innate cellular immune parameters analysed. The *in vivo* study tested diets with 0.5 and 1% inulin, and showed a significant inhibition of leucocytes phagocytosis and respiratory burst at the first week of feeding. These results led the authors to conclude that inulin does not seem to be a good immunostimulant for gilthead sea bream. However, more recent studies seem to evidence the contrary. Cerezuela et al. (2012) fed fish

with 1, 1.5, and 3% inulin and, based on improvements of complement activity and of phagocytic ability and capacity, the authors concluded that 1% was the best level of inulin incorporation to the diets. In a second trial, inulin increased the complement activity at 2 and 4 weeks, while serum immunoglobulin M, respiratory burst, and phagocytic ability and capacity were only higher at week two. Fish fed inulin also had higher survival after a challenge with *P. damselae* subsp. *piscicida*. On the contrary, inulin did not affect the expression of immune related genes in the head kidney. Subsequently, Cerezuela et al. (2013a) reported the effect of inulin in intestinal morphology and microbiota. Fish fed inulin presented signs of gut oedema and inflammation, somewhat similar to that observed in fish fed SBM. Moreover, gut bacterial richness was also lower in fish fed inulin. Cerezuela et al. (2013b) further studied the effect of inulin on genes involved in inflammation, and concluded that this prebiotic may modulate intestinal gene expression. Data on gene expression, together with previous data (Cerezuela et al., 2012; Cerezuela et al., 2013a), allowed the authors to conclude that gut alterations in fish fed inulin correlated with the slight inflammation mediated by IL-8. Moreover, inulin supplementation seems to reinforce the junctions between enterocytes, as supported by the expression of β-actin and occludin, and the transport of iron molecules. From the above results, it seems that inulin is capable of modulating gilthead sea bream immune response, but results do not seem promising since effects in the gut seem to indicate that inulin can compromise body homeostasis, which is mainly maintained by the epithelial lining of the GI tract.

In Table 7 a detailed review of the prebiotics already tested in gilthead sea bream and the main results observed is presented.

Table 7 – Prebiotics use in gilthead sea bream.

Prebiotic	**Fish weight (g)**	**Dosage and administration length**	**Results**	**References**
Bio-MOS®	≈172g	0.2 and 0.4% - 12 weeks	↗growth, protein, carbohydrate and energy digestibility →survival, body composition, lipids digestibility ↘FCR	Gültepe et al. (2011)
Bio-MOS®	≈172g	0.2 and 0.4% - 12 weeks	→RBC, WBC, Thr, Ht, Hb, MCH, MCHC, MCV	Gültepe et al. (2012)
Bio-MOS®	172.11±13.19g	0.2% - 90 days	↗serum urea →serum AST, ALT, ALP, PO_4, Fe, Na, K, Mg, Ca, Cl, TP, GLC, CHOL, TRIG, CREA, DBIL, IBIL, URICA	Gültepe et al. (2015)
MOS	≈ 24g	0.2 and 0.4% in FM-based diets - 9 weeks	↗AG microvilli density and length, PG absorptive surface (0.4%), microvilli density (0.2 and 0.4%) and length (0.4%), gut microbiota richness and diversity →growth, FCR, body composition, liver glycogen, AG absorptive surface ↘CF (0.2%), HSI	Dimitroglou et al. (2010b)

Prebiotic	Fish weight (g)	Dosage and administration length	Results	References
			(0.4%)	
MOS	≈ 24 g	0.4% in SBM-based diets - 9 weeks	↗AG microvilli density, PG microvilli density and length →growth, FCR, body composition, CF, HSI, liver glycogen, AG absorptive surface and microvilli length, PG absorptive surface, gut microbiota richness and diversity	Dimitroglou et al. (2010b)
Inulin	≈175g	62.5, 125, 250, 500 or 1000mg ml^{-1} - 30, 90, 180 or 300 min or 24h(*in vitro*)	→leucocyte viability, phagocytic ability and capacity, respiratory burst, cytotoxicity, peroxidase activity	Cerezuela et al. (2008)
Inulin	≈175g	0.5 and 1% - 1 or 2 weeks	→serum peroxidase, complement, phagocytic ability and leucocyte peroxidase (1 and 2 weeks), phagocytic capacity and respiratory burst (2 weeks) ↘ phagocytic capacity (1%) and respiratory burst (0.5%) both at 1 week	Cerezuela et al. (2008)
Inulin	≈ 50 g	1, 1.5 and 3% - 2 and 4 weeks	↗complement (1%), phagocytic ability (1%), phagocytic capacity (1 and 3%, only at 2 weeks) ↘ phagocytic capacity (3% at 4 weeks)	Cerezuela et al. (2012)
Inulin	≈ 50 g	1% - 2 and 4 weeks	↗complement (2 and 4 weeks), IgM, respiratory burst, phagocytic ability and capacity (2 weeks), survival after *P. damselae* subsp. *piscicida* challenge → IgM, respiratory burst, phagocytic ability and capacity (4 weeks), expression of IgMh, TCRβ, MHCIα, MHCIIα, CSF-1R, β-def	Cerezuela et al. (2012)
Inulin	≈ 50 g	1% - 4 weeks	↗Vh, Gd, GC PAS+, IELs, intercellular space, enterocyte vacuolisation, microvilli disruption/damage →Va, Wa, La, Gd:Vh, LPLs, gut bacteria diversity ↘GC, GC PAS+AB+, MVh, gut bacteria richness	Cerezuela et al. (2013a)
Inulin	≈ 50 g	1% - 4 weeks	↗expression of IL-8, β-actin, Ocl, Tf →expression of IL-1, IL-6, CASP-1, COX-2, ZO-1, α-Tub, Vim, Tricel, Am, Trip, ALP, PepT-1	Cerezuela et al. (2013b)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. AG: anterior gut; ALP: alkaline phosphatase; ALT: alanine amino transferase; Am: α-amylase; AST: aspartate amino transferase; Ca: calcium; CASP: caspase; CF: condition factor; CHOL: cholesterol; Cl: chloride; COX: cyclooxygenase; CREA: creatinine; CSF-1R: colony-stimulating factor receptor 1; DBIL: direct bilirubin; FCR: feed conversion ratio; Fe: iron; FM: fish meal; GC: goblet cells; GC PAS+: goblet cells PAS+; GC PAS+AB+: goblet cells PAS+ and AB+; Gd: gut diameter; Gd:Vh ratio villus height:gut diameter; GLC: glucose; Hb: haemoglobin; HSI: hepatosomatic index; Ht: haematocrit; IBIL: indirect bilirubin; IELs: intraepithelial leucocytes; IgM: immunoglobulin M; IgMh: immunoglobulin M (heavy chain); IL: interleukin; K: potassium; La: intestinal lumen area; LPLs: lamina propria leucocytes; MCH: mean cellular haemoglobin; MCHC: mean cellular haemoglobin concentration; MCV: mean cellular volume; Mg: magnesium; MHCIα: major histocompatibility complex class Iα; MHCIIα: major histocompatibility complex class IIα; β-def: β-defensin; MVh: microvillus height; Na: sodium; Ocl: occludin; PepT: peptide transporter; PG: posterior gut; PO_4: phosphate; RBC: red blood cells; SBM: soybean meal; TCRβ: T-cell receptor β; Tf; transferrin; Thr: thrombocytes; TP: concentration of total protein; Tricel: tricelulin; TRIG: triglyceride; Trip: trypsin; Tub: tubulin; URICA: uric acid; Va: villus area; Vh: villus height; Vim: vimentin; Wa: intestinal wall area; WBC: white blood cells; ZO: zona-occludens.

1.3.3 European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758)

European sea bass (Figure 9) is a eurythermic (2-32°C) and euryhaline fish of the Moronidae family (FAO, 2005c; Hidalgo and Alliot, 1988). It is a species with a demersal behaviour, commonly inhabiting shallow waters but that can also be found in coastal waters up to 100m depth, estuaries, brackish water lagoons, and even rivers. European sea bass can be found in the Mediterranean and the Black Sea, and also in the North Atlantic from Norway and the British Isles southward to Morocco, Canaries islands, and Senegal. Spawning occurs between January and March in the Mediterranean and Black Sea, and from March to June in the British Isles. Breeding takes place near to river mouths and estuaries or in littoral areas (FAO, 2005c).

European sea bass was primary cultured in coastal lagoons and tidal reservoirs. Only in the 1960s intensive production begun in France and Italy, and by the late 1970s most Mediterranean countries produced European sea bass. This was the first marine non-salmonid species to be intensively produced in Europe. Nowadays, European sea bass is exploited in most countries bordering the Mediterranean Sea, with Greece, Turkey, Italy, Spain, Croatia, and Egypt being the main producers (FAO, 2005c). The production has oscillated through the years, with a total production of 161 059 tonnes (representing $ 1 034 400 000) in 2013, with 489 tonnes ($ 4 078 000) of European sea bass being produced in Portugal (FIGIS, 2015).

Although European sea bass is a well-established aquaculture species, heavy losses due to disease outbreaks may occur due to bacterium (*Vibrio spp., P. damselae* subsp. *pasteurella, Flexibacter maritimus, Mycobacterium marinum, Chlamydia*-like), virus (Nodavirus), ciliates (*Cryptocaryon irritans, Philasterides dicentrarchi, Uronema* sp., *Tetrahynema* sp.), nematodes (*Anisakis* spp.), dinoflagellates (*Amyloodinium occelatum*), myxosporidia (*Shaerospora dicentrarchi, S. testicularis, Ceratomyxa labraci*), microsporidia (*Glugea* sp.), monogenean trematode (*Diplectanum aequans, D. laubieri*) and crustaceans (*Ceratothoa oestroides, Nerocilla orbiguyi, Anilocra physoides*) (FAO, 2005c).

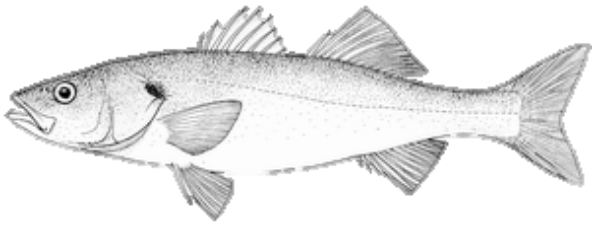

Figure 9 – European sea bass (*Dicentrarchus labrax*)
(FAO, 2005c)

1.3.3.1 Nutritional recommendations

European sea bass is a carnivorous fish, which feeds on small fish and invertebrates such as crustaceans and molluscs (FAO, 2005c). Table 8 presents a summary of the dietary macronutrient recommendations for European sea bass.

The first studies about protein requirements reported dietary requirements of 52 to 60% (Alliot et al., 1974; Metailler et al., 1981). Thereafter, other studies demonstrate that fish performed equally well with dietary protein levels between 48 to 54% (Ballestrazzi et al., 1994; Hidalgo and Alliot, 1988; Peres and Oliva-Teles, 1999b) or even lower, 43 to 45% (Dias et al., 1998; Pérez et al., 1997), depending on the digestible protein to digestible energy ratio of the diet. In fact, based on nutrient and energy utilization data, a diet with 40% protein and 27% starch could be a good standard diet for European sea bass (Hidalgo and Alliot, 1988).

Based on dose-response studies, requirements (in g/16g N) of 3.9g for arginine (Tibaldi et al., 1994), 4.8g for lysine, (Tibaldi and Lanari, 1991), 2g for methionine (Thebault et al., 1985), 0.5g for tryptophan (Tibaldi et al., 1993), and 2.3-2.6g for threonine (Tibaldi and Tulli, 1999) were estimated. For the other EAA there are no dose-response estimations, but indirect estimations using the ideal protein method estimated requirements (in g/16g N) of 2.6g for phenylalanine + tyrosine, 1.6g for histidine, 2.6g for isoleucine, 4.3g for leucine and 2.9g for valine (Kaushik, 1998). More recently, Peres and Oliva-Teles (2006) estimated the optimum EAA to non-EAA ratio of the diets for this species, and conclude that a ratio of 50/50 is necessary to maximise growth performance, and of 60/40 is necessary to maximise feed, protein and energy utilization.

Alliot et al. (1974), reported highest growth rates of European sea bass fed diets with 12% lipids. In accordance, other authors also reported no beneficial effects of increasing dietary lipid levels above 12% (Metailler et al., 1981; Peres and Oliva-Teles, 1999a; Pérez et al., 1997). On the other hand, some authors report improved growth when fish were fed diets with 18-19% lipids, although an increase in body fat was also observed (Dias et al., 1998; Lanari et al., 1999). More recently, Boujard et al. (2004) further reported that European sea bass fed diets with 30% lipids had the same growth than fish fed diets with 10% lipids, but with lower FI. Moreover, with the same FI, growth and body lipid content were higher in fish fed 30% lipids.

Few studies are available regarding HUFA requirements of European sea bass juveniles. Coutteau et al. (1996) indicated that during and immediately after weaning n-3 HUFA requirements do not exceed 1% of the dry diet. For 14g European sea bass fed diets with 18% lipids, the n-3 HUFA requirements were reported to be 0.7%, with a DHA:EPA ratio of 1.5:1 (Skalli and Robin, 2004).

Hidalgo and Alliot (1988) reported that diets with 15% gelatinized maize starch and 50% protein produced the best growth and FE of European sea bass, and that an incorporation of 27% starch allowed to reduce dietary protein to 40%, leading to better nutrient and energy utilization. In addition, Pérez et al. (1997) stated that carbohydrates level in European sea bass diets should not exceed 30%. Although native or waxy maize starch at dietary levels of 10 or 20% did not affect fish growth rate, starch digestibility was higher for waxy starch, and decreased with increasing dietary starch levels (Enes et al., 2006b).

Since there is not enough information available on vitamin requirements, it was suggested that the levels established for salmonids could be used in practical diets for European sea bass. However, in semi-purified diets slightly higher levels were necessary to allow satisfactory growth rates (Kaushik et al., 1998). Fournier et al. (2000) studied the vitamin C requirements of European sea bass, and showed that a minimum of 5mgKg^{-1} diet should be used to maximise growth, although higher levels were required based on whole body hydroxyproline (31mgKg^{-1}) and liver ascorbic acid concentrations (121mgKg^{-1}).

Regarding mineral requirements, the only available study refers to phosphorus and reports a requirement of 0.65% of dietary phosphorus (Oliva-Teles and Pimentel-Rodrigues, 2004).

Table 8 – Summary of the main nutritional groups (protein, lipids and carbohydrates) recommendations European sea bass.

Feeding behaviour	**Nutrient**	**Fish weight (g)**	**Recommendation level**	**References**
Carnivorous	Protein	18g	52%	Alliot et al. (1974)
		≈75g	60%	Metailler et al. (1981)
		31-37g	50%	Hidalgo and Alliot (1988)
		31-37g	40% protein if 27% starch included	Hidalgo and Alliot (1988)
		75.8±5.8g	49-54%	Ballestrazzi et al. (1994)
		2.78g	45%	Pérez et al. (1997)
		5.9±0.1g	43%	Dias et al. (1998)
		5.6g	48%	Peres and Oliva-Teles (1999b)

Feeding behaviour	Nutrient	Fish weight (g)	Recommendation level	References
	Lipids	18g, ≈75g and ≈7g, respectively	12%	Alliot et al. (1974); Metailler et al. (1981); Peres and Oliva-Teles (1999a)
		2.78g	12-14%	Pérez et al. (1997)
		5.9±0.1g	18%	Dias et al. (1998)
		91.5±5.7g	19%	Lanari et al. (1999)
		≈220-260g	30%	Boujard et al. (2004)
	Carbohydrates	31-37g	27% gelatinized maize starch	Hidalgo and Alliot (1988)
		2.78g	30% carbohydrates	Pérez et al. (1997)
		23.3g	Up to 20% native or waxy maize starch	Enes et al. (2006b)

1.3.3.2 Fish meal replacement by plant feedstuffs

In European sea bass, a replacement of 95% of dietary FM by a PF mixture (corn gluten meal, wheat gluten, extruded wheat, SBM and rapeseed meal) and lysine supplementation, was already accomplished without affecting fish growth, diet digestibility, or voluntary FI (Kaushik et al., 2004). Tibaldi et al. (2006) showed that the use of a diet with 50% enzyme-treated SBM supplemented with methionine did not affect fish growth or feed digestibility when compared with a FM-based diet. However, a diet with 60% SBM inclusion level reduces both growth and feed digestibility.

1.3.3.3 Prebiotics use

Up to now, MOS (Torrecillas et al., 2007; Torrecillas et al., 2011a; Torrecillas et al., 2011b; Torrecillas et al., 2012; Torrecillas et al., 2013; Torrecillas et al., 2015a; Torrecillas et al., 2015b) and XOS were the only prebiotics assessed in European sea bass (Abdelmalek et al., 2015).

Torrecillas et al. (2007) fed European sea bass with 0.2 and 0.4% MOS from Bio-MOS® and observed that fish fed MOS had higher growth and presented lower liver lipid vacuolization and regular-shaped hepatocytes around sinusoidal spaces. Moreover, fish fed 0.4% MOS had higher phagocytic index, and both MOS levels decreased the number of infected fish after a challenge with *V. alginolyticus*. Thus, the authors concluded that MOS at 0.4% enhances growth, activates immune system, and increases fish resistance against bacterial infection directly inoculated in the gut, one of the main sites of infection in fish (Torrecillas et al., 2007).

Then, Torrecillas et al. (2011b) tested MOS at 0.2, 0.4, and 0.6% and demonstrated that MOS can modify lipid metabolism. MOS affected fish liver in the same way as reported by Torrecillas et al. (2007). The higher number of acid mucins secreting cells observed in fish fed MOS may improve resistance to bacterial infections. Authors concluded that MOS supplemented at 0.4 and 0.6% improved FCR, activated fish immune system, and increased gut mucus secretion, without affecting sensorial parameters or the biochemical composition of flesh (Torrecillas et al., 2011b).

Torrecillas et al. (2011a) reported that 0.4% MOS fed to European sea bass increased anterior gut mucosal folds height, width and surface area. Although posterior gut presented shorter folds, they were wider, thus resulting in increased total surface area. In addition, gut cells secreting acid mucins and the density of eosinophilic granulocytes in the mucosa were higher in fish fed MOS. This, together with an improvement in gut mucus lysozyme activity could be related to the reduced *in vivo* and *ex vivo* gut bacterial translocation found (Torrecillas et al., 2011a). Furthermore, dietary supplementation with 0.4% MOS reduced fish mortality after challenged with *V. anguillarum* or *V. anguillarum* plus confinement stress. Posterior gut of fish fed MOS presented similar microbial profiles in stressed and non-stressed fish, whereas microbial profiles of stressed and non-stressed fish fed the control diet were different, indicating that MOS reduced stress on microbiota diversity (Torrecillas et al., 2012). Taking into consideration all the above information, it can be assumed that a general reinforcement of the innate immune system, particularly of the intestinal barrier efficiency, is the main defence mechanism against pathogenic microorganisms of European sea bass fed MOS (Torrecillas et al., 2012).

Fish fed 0.4% MOS also had increased prostaglandins production and reduction of posterior gut neutral lipid fraction, mainly due to a reduction of triacylglycerol content. On the contrary, the polar lipid fraction increased in fish fed MOS, mainly due to an increase in phosphatidylethanolamine and phosphatidylcoline contents. Transmission electron microscopy of fish posterior gut also revealed a healthier gut. Data indicates that MOS enhanced fish posterior gut epithelial defences by increasing membrane polar lipids content in relation to a stimulation of the eicosanoid cascade and GALT, promoting posterior gut health status (Torrecillas et al., 2013).

Recently, Torrecillas et al. (2015b) tested the effect of 0.16% concentrated MOS (cMOS) on European sea bass. Dietary cMOS supplementation altered especially liver and muscle fatty acid profiles by reducing the level of fatty acids that are preferential substrates for β-oxidation, in spite of a preferential retention of LC-PUFA. Posterior gut had lower width, and no effects on the number of cells secreting acid mucins were observed in fish fed cMOS. The authors concluded that cMOS increased specific growth rate, stimulated selected cellular gut-associated immune system parameters, and affected lipid metabolism in liver and muscle, increasing LC-PUFA accumulation and promoting β-oxidation.

Also recently, Torrecillas et al. (2015a) evaluated the effect of 0.4% MOS on diets with fish oil (FO) or with FO replaced by soybean oil (SBO). MOS supplemented to diets with SBO

decreased hepatocytes area and decreased liver lipids. Whereas MOS supplemented to FO based diets increased muscle lipids content. Dietary MOS favoured liver but not muscular n-3 PUFA, DHA, EPA, and ARA deposition in the FO diet but not in the SBO diet. The authors concluded that MOS favours fish performance and helps to minimize side effects on liver lipid accumulation and hepatocyte vacuolization derived from high dietary SBO levels.

In another recent study, Abdelmalek et al. (2015) tested the effect of 0.5 and 1% XOS in European sea bass fingerlings. Fish fed XOS presented higher weight, while FE and PER were only improved in fish fed 0.5% XOS. Both before and after being challenged with *A. hydrophila*, fish fed XOS presented improved immune parameters and had higher survival to the bacterial challenge. The authors concluded that XOS included in the diet at 0.5% increases fish growth, stimulates immunity, and improves resistance to infection by *A. hydrophila* directly inoculated in the gut.

In Table 9 a detailed review of the prebiotics already tested in European sea bass and the main results observed is presented.

Table 9 – Prebiotics use in European sea bass.

Prebiotic	**Fish weight (g)**	**Dosage and administration length**	**Results**	**References**
Bio-MOS®	33.75±7.69g	0.2 and 0.4% - 67 days	↗growth, phagocytic index (0.4%) →CF, FCR, FI, body composition, body saturated and monosaturated FA, Σn-3, Σn-6, Σn-9, Σn-3 HUFA, hepatocytes maximum longitude, lysozyme, ACH50, gut morphology ↘hepatocytes minimum longitude and area (0.4%), infected fish after exposure to *V. alginolyticus*	Torrecillas et al. (2007)
Bio-MOS®	60.64±0.85g	0.2, 0.4 and 0.6% - 60 days	↗PG cells secreting acid mucins and phagocytic index (0.4 and 0.6%) →growth, CF, body ash and protein, body saturated and monosaturated FA, Σn-3, Σn-6, Σn-9, Σn-3 HUFA, AG cells secreting acid mucins, serum lysozyme, protein and lipid digestibility ↘FCR and FI (0.4 and 0.6%), G6PD, ME (0.2 and 0.4%), body lipids (0.6%), body moisture (0.4 and 0.6%), hepatocytes area, maximum and minimum longitude	Torrecillas et al. (2011b)
Bio-MOS®	60.64±0.85g	0.2, 0.4 and 0.6% - 14 months	↗PG cells secreting acid mucins →AG cells secreting acid mucins, fillet protein, lipids and ash, organoleptic parameters ↘fillet moisture (0.2%)	Torrecillas et al. (2011b)

Prebiotic	Fish weight (g)	Dosage and administration length	Results	References
Bio-MOS®	≈116g	0.4% - 8 weeks	↗AG folds height, width, surface area and cells secreting acid mucins, PG folds width, area and cells secreting acid mucins, AG and PG lamina propria engrossment, gut mucus lysozyme →rectum folds width, skin mucus lysozyme, gut and skin mucus bactericidal activity ↘PG folds height, rectum folds height and area, AG and PG bacterial translocation	Torrecillas et al. (2011a)
Bio-MOS®	45.95±0.60g	0.4% - 8 weeks	↗growth, CF, cortisol in stressed non-inoculated fish after confinement stressor (at 4h) →*V. anguillarum* presence in liver after the *V. anguillarum* challenge and stress confinement, *V. anguillarum* presence in head kidney after the *V. anguillarum* challenge ↘mortality after infection with *V. anguillarum* and confinement stressor, *V. anguillarum* presence in head kidney after the *V. anguillarum* challenge plus confinement stressor (at 4h), cortisol after the *V. anguillarum* challenge and confinement stressor (at 4h)	Torrecillas et al. (2012)
Bio-MOS®	45.95±0.60g	0.4% - 8 weeks	↗growth, CF, PG cholesterol/sterols, phosphatidylethanolamine, PS, PC, total polar lipids, gut FA 14:0 and 16:1n-7 (PS), 22:5n-3 (PC), 24:0 and total n-6 PUFA (LPC) ↘ PG triacylglycerols, total neutral lipids, gut FA 15:0 (PHI), 22:5n-6 and 20:5n-3 (PS), 14:0 (LPC)	Torrecillas et al. (2013)
cMOS	20.62±0.33g	0.16% - 8 weeks	↗fish length, SGR, expression of MCHII, TCRβ, Ig and Casp-3 →fish weight, CF, HSI, VSI, muscle, liver, AG and PG composition, PG length, cells secreting acid mucins, expression of IL-1β, IL-10, TNFα, IL-8, IL-6, $CD4^+$, $CD8\alpha^+$, MHCI, COX2, Casp-9, LPL, FABP7, ANGPTL3 and HMGRC ↘expression of TGFβ and FADS2, PG width	Torrecillas et al. (2015b)
Bio-MOS®	20.63±0.12g	0.4% (in FO or SBO-based diets) - 8 weeks	↗liver protein (SBO), muscle lipids (FO) →growth, CF, HSI, VSI, hepatocytes minimum and maximum length, muscle protein, liver protein (FO), muscle lipids (SBO), liver lipids (FO), AG lipids, muscle and liver ash ↘hepatocytes area and liver	Torrecillas et al. (2015a)

Prebiotic	Fish weight (g)	Dosage and administration length	Results	References
			lipids (SBO)	
XOS	4.75±0.69g	0.5 and 1% - 12 weeks	↗growth, FE (0.5%), PER (0.5%), survival after challenge with *A. hydrophila* (1%), RBC pre and pos-C, WBC pre-C, Hb pre and pos-C, Ig pre-C, Ig pos-C (0.5%), lysozyme pre and pos-C →liver weight, HSI, liver morphology ↘WBC pos-C, serum protein pre-C (1%)	Abdelmalek et al. (2015)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the prebiotic relative to the control. In the case of prebiotic being tested at more than one level, and when not indicated within brackets, it means that all levels had the same effect. ACH50: alternative complement pathway activity; AG: anterior gut; ANGPTL: angiopoietin-like proteins; $CD4^+$: CD4 molecule; $CD8\alpha^+$: CD8α molecule; CF: condition factor; COX: cyclooxygenase; FA: fatty acid; FABP: fatty acid binding protein; FADS: fatty acid desaturase; FCR: feed conversion ratio; FE: feed efficiency; FI: feed intake; FO: fish oil; G6PD: glucose-6-phosphate dehydrogenase; Hb: haemoglobin; HMGRC: 3-hydroxy-3-methylglutaryl-CoA reductase; HSI: hepatosomatic index; HUFA: highly unsaturated fatty acids; Ig: immunoglobulin; IL: interleukin; LPC: lysophosphatidylcholine; LPL: lipoprotein lipase; ME: malic enzyme; MHCI: major histocompatibility complex class I; MHCII: major histocompatibility complex class II; PC: phosphatidylcholine; PER: protein efficiency ratio; PG: posterior gut; PHI: phosphatidylinositol; pos-C: pos-challenge; pre-C: pre-challenge; PS: phosphatidylserine; PUFA: polyunsaturated fatty acids; RBC: red blood cells; SBO: soybean oil; TCRβ: T-cell receptor β; TGFβ: transforming growth factor-β; TNF: tumor necrosis factor; VSI: viscerosomatic index; WBC: white blood cells.

1.3.4 White sea bream (*Diplodus sargus*, Valenciennes, 1830)

White sea bream (Figure 10) is a benthopelagic fish of the Sparidae family. It is a species with demersal behaviour, inhabiting rocky and sand bottoms up to depths of 150m, and it is also abundant in shallow waters. Commonly found in the Mediterranean Sea, Atlantic coast from the Bay of Biscay to Cape Verde, and southwards to Angola, South Africa to Madagascar, including Madeira, Canaries, Cape Verde, Ascension, and St. Helena islands. There is no information available about white sea bream temperature tolerance, however a study reported fish catches in waters with temperatures varying between 13 to 25°C. Spawning occurs between January and March in the eastern Mediterranean, and between March and June in the western Mediterranean (Abellán and Basurco, 1999; FAO, 2005d; Golomazou et al., 2006).

The first records of production date from the earlies 1970s, but production only increased in the 1990s. The production has oscillated through the years, with a total production of 24 tonnes (representing $ 176 000) in 2013, with no production reported for white sea bream in Portugal, except for one tonne reported as being produced in 2009 (FIGIS, 2015).

Diseases affecting with sea bream are mainly related with bacterium (*V. anguillarum, V. alginoliticus*), ectoparasites (Monogenea, Digenea), and endoparasites (Myxosporida) (Golomazou et al., 2006).

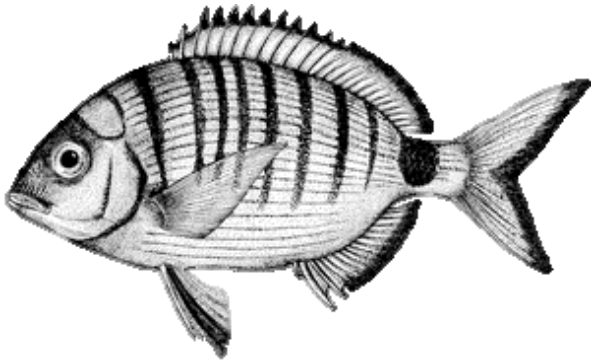

Figure 10 – White sea bream (*Diplodus sargus*)
(FAO, 2005d)

1.3.4.1 Nutritional recommendations

White sea bream is considered an omnivorous fish at early stages, feeding on seaweeds and small larvae, and changes to a carnivorous feeding behaviour at the adult stage, feeding on benthic invertebrates such as worms, molluscs, and crustaceans (Abellán and Basurco, 1999). Nonetheless, white sea bream feeds mostly on algae and echinodermata, followed by barnacles, worms, and gastropods. At lower quantities they also predate fish eggs, amphipods, gastropods, fish, tunicates, decapods, bivalves, and others invertebrates (Figueiredo et al., 2005). Therefore, white sea bream can be considered an omnivorous species, with an opportunistic behaviour.

Knowledge of the nutritional requirements of this species is limited. Table 10 presents a summary of the dietary macronutrient recommendations for white sea bream. In a study that tested two dietary protein levels, 15 and 28%, and two lipids levels, 12 and 16%, fish performed better when fed the 28% protein diets, independently of the lipid level used (Ozório et al., 2006). Sá et al. (2008a) tested an increasing range of dietary protein levels, ranging from 6 to 49%, and concluded that for maximum growth performance fish should be fed a diet with 27% protein, while for maximum protein retention a dietary protein level of 33% should be used.

No data is available regarding white sea bream juveniles EAA requirements. The only study available was done with larvae, where the EAA profile of fish carcass was used as indicator of fish amino acids requirements at several larval ages (Saavedra et al., 2006).

Data on lipids utilization by white sea bream seems to indicate no protein sparing effect due to dietary lipids (Ozório et al., 2006; Sá et al., 2006, 2008b). Fish with initial body weights of 11 or 41g performed equally well with diets including 12 or 16-18% lipids; however, lipid content of the viscera, liver, and muscle increased in the smaller fish when fed the 16% lipid diet (Ozório et al., 2006; Sá et al., 2006). Sá et al. (2008b) observed no advantages of increasing dietary lipids level above 9% in 17g fish, since up to a dietary lipid of 24% no growth improvement was observed. However, in a study with fry of an initial body weight of 1.5g, growth depression was observed when dietary lipid level increased from 12 to 18% (Sá et al., 2006).

EFA requirements of the Sparidae family where only determined for gilthead sea bream and for red sea bream (*Pagrus major*) (Oliva-Teles et al., 2011), no data being available for white sea bream.

White sea bream can utilize diets with 36% waxy maize starch, this allowing a reduction of dietary protein from 64% to 38% without negatively affecting fish performance (Sá et al., 2007). Despite normal maize starch appears to be more efficiently used as energy source than waxy starch, both starches can be incorporated in the diets up to 42% without affecting growth (Sá et al., 2008c).

Vitamins and minerals requirements of white sea bream where not yet studied.

Table 10 – Summary of the main nutritional groups (protein, lipids and carbohydrates) recommendations for white sea bream.

Feeding behaviour	Nutrient	Fish weight (g)	Recommendation level	References
Omnivorous fish at early stages changing to carnivorous when adult	Protein	10.7±0.2g	28%	Ozório et al. (2006)
		≈22g	27-33%	Sá et al. (2008a)
	Lipids	10.7±0.2g	12-16%	Ozório et al. (2006)
		≈41g	12-18%	Sá et al. (2006)
		≈1.5g	12%	Sá et al. (2006)
		≈17g	9-24%	Sá et al. (2008b)
	Carbohydrates	≈14g	Up to 36% waxy maize starch	Sá et al. (2007)
		≈14g	Up to 42% normal or waxy maize starch	Sá et al. (2008c)

1.3.4.2 Fish meal replacement by plant feedstuffs

There is a lack of studies about PF utilization in white sea bream. The use of diets with up to 42% of starch, without impairing growth performance, point to an adequate PF use by this species (Sá et al., 2007; Sá et al., 2008c). Moreover, the omnivorous feeding habit of white sea bream further supports this supposition. However, in a study by Cardoso (2010), substitution

of only 50% of the FM by PF (wheat meal, corn gluten and SBM) was enough to decrease fish growth, although only a substitution of 100% of the FM in the diet led to mid gut and liver histological deleterious modifications. The author suggested that the worst results obtained with PF inclusion might be connected with a possible deficiency of taurine in the diets.

1.3.4.3 Prebiotics use

Only one study is available on the use of prebiotics in white sea bream. Dimitroglou et al. (2010a) studied the effect of 0.2% MOS, fed during 43 days, on the development, gut integrity, and quality of white sea bream larvae. MOS supplementation had no effect on larvae growth or survival, however it led to a 12% increase in villi surface area and a 26% increase in microvilli length. Moreover, survival after hypo-saline or hyper-saline challenges was higher in MOS fed larvae. The authors hypothesised that the increased survival after the saline challenges in larvae fed MOS may be connected with the improved gut morphology, since the gut of marine fish has an important role in osmoregulation.

1.4 Aims and thesis overview

Several studies have shown the beneficial effects of using prebiotics in fish. However, some prebiotics and species have been scarcely studied. Within the less studied species, there are species already produced and others that are referred as important for diversification of Mediterranean aquaculture. Moreover, some prebiotics used in mammals with very promising results are still to be properly studied in fish. Therefore, the following studies were carried out to increase the knowledge on selected prebiotics, namely scFOS, XOS, and GOS, in turbot, gilthead sea bream, European sea bass, and white sea bream juveniles. Topics such as growth performance, feed utilization, intermediary metabolism, gut microbiota composition, gut histomorphology, digestive enzymes, immunology, and oxidative status, were evaluated.

Thus, in Chapter 2, Chapter 3, and Chapter 4, the effect of scFOS was tested at three incorporation levels, 0.5, 1, and 2%, in turbot reared at two temperatures, 15 and 20°C, which are temperatures representative of winter and summer conditions, respectively. Prebiotics were incorporated in diets whose protein was provided at 50:50 from FM and PF. In Chapter 2 data is presented on the prebiotic and temperature effects in growth performance, feed utilization efficiency, and intermediary metabolism. In Chapter 3, the prebiotic and temperature effects on allochthonous microbiota, digestive enzymatic activity and gut histomorphology were evaluated. In Chapter 4, prebiotic and temperature effects were assessed in fish hepatic oxidative status and immune response.

Thereafter, scFOS was tested at 0.1, 0.25, and 0.5%, in gilthead sea bream reared at 18 and 25°C, representatives of suboptimal and optimal rearing temperatures, respectively. Prebiotics were incorporated in diets whose protein was provided at 50:50 from FM and PF. In Chapter 5, the effects of scFOS and temperature on growth performance, feed utilization efficiency, and

intermediary metabolism was evaluated. Chapter 6 presents data regarding fish immunological status, gut microbiota, digestive enzymes activities, and gut histomorphology.

Subsequently, in Chapter 7 and Chapter 8, the effects of scFOS and XOS at 1% incorporation level were tested in European sea bass. More challenging diets, with higher incorporation levels of protein from PF were used, namely 70% of protein from PF. The two prebiotics were also tested in a FM-based diet in order to compare with the results of the PF-based diets. Thus, in Chapter 7 the effects of scFOS and XOS on growth performance, feed utilization efficiency, and intermediary metabolism were evaluated. In Chapter 8 data on the effect of the prebiotics on fish gut morphology and hepatic oxidative status is presented.

In Chapter 9 and Chapter 10 we report a study with white sea bream where one more prebiotic, GOS, was tested together with scFOS and XOS, all incorporated at 1% in diets with 70% of the protein coming from PF. Chapter 9 presents results of growth performance, feed utilization efficiency, intermediary metabolism, gut microbiota, and digestive enzymes, and in Chapter 10 the effects of prebiotics on the immune and hepatic oxidative status, and on gut morphology are described.

Chapter 2
Effects of short-chain fructooligosaccharides on growth performance and hepatic intermediary metabolism in turbot (*Scophthalmus maximus)* reared at winter and summer temperatures

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Effects of short-chain fructooligosaccharides on growth performance and hepatic intermediary metabolism in turbot (*Scophthalmus maximus*) reared at winter and summer temperatures

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Abstract

The effect of short-chain fructooligosaccharides (scFOS) incorporation on growth, feed utilization, body composition, plasmatic metabolites and selected liver enzyme activities of turbot juveniles reared at winter (15 °C) and summer (20 °C) temperatures was studied. Four comparable diets were formulated to contain circa 50 : 50 fish meal and plant ingredients as protein sources. Experimental diets included increasing levels of scFOS (0, 5, 10 and 20 g kg^{-1}). Final weight was higher at 20 °C, but thermal growth unit, feed efficiency, nitrogen and energy retention were better at 15 °C. scFOS supplementation did not affect fish growth performance. Fish reared at 15 °C had higher liver glycogen, visceral and hepatosomatic indices. Liver lipids, plasma triglycerides, total lipids, cholesterol HDL and LDL were higher in turbot reared at 20 °C. Malic enzyme, fatty acid synthetase, alanine aminotransferase and glutamate dehydrogenase activities were higher in fish reared at 15 °C. Malic enzyme was lower in turbot fed with 20 g kg^{-1} scFOS compared to control diet; however, fatty acid synthetase presented an increasing trend as dietary scFOS increased up to 10 g kg^{-1}. Glutamate dehydrogenase activity was higher in fish fed the control diet. Results seem to indicate no benefits of scFOS incorporation to diets on growth performance of turbot.

KEY WORDS: amino acid catabolism enzymes, lipogenic enzymes, plasmatic metabolites, prebiotics, temperature, turbot

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Introduction

The aquaculture industry has grown considerably over the last three decades, between 1980 and 2010 world aquaculture production grew at an average rate of 8.8% per year (FAO 2012). One of the bottlenecks for the sustainable development of the aquaculture industry is the incidence of infectious diseases and ineffective methods to combat disease outbreaks. Antibiotics were usually applied as growth promoters and for disease prophylaxis; however, the EU moratorium (Regulation (EC) No 1831/2003) banned the use of antibiotics for these functions. To overcome this restriction, prebiotics have been considered as an environmentally friendly alternative to prevent disease and promote growth (Ringø *et al.* 2010; Dimitroglou *et al.* 2011).

Prebiotics are defined as non-digestible feed ingredients that are beneficial by promoting growth and/or activity of selected bacteria within the host gastrointestinal tract (Gibson & Roberfroid 1995). Prebiotics are not digested by fish but are fermented by gut microbiota, resulting in the acidification of gut content and in production of short-chain fatty acids which may be used by the enterocytes as an energy source or for stimulating the growth of beneficial bacteria including lactic acid bacteria (LAB) (Merrifield *et al.* 2010). Prebiotics mostly consist of oligosaccharides, and among them, mannanoligosaccharides (MOS), fructooligo-

saccharides (FOS, or oligofructose) and inulin are the best studied in fish (Ringø *et al.* 2010; Dimitroglou *et al.* 2011).

Studies on prebiotics in fish are somewhat contradictory with some studies reporting beneficial effects with the use of prebiotics, while others report an absence of apparent effects. Reported beneficial effects of prebiotics in fish include improved growth rate, feed efficiency, feed digestibility, survival, immunological status and resistance to bacterial and viral diseases, mainly due to modulation of the intestinal microbiota (Merrifield *et al.* 2010; Ringø *et al.* 2010; Dimitroglou *et al.* 2011).

Fructooligosaccharides (FOS) are prebiotics commonly used in humans and farm animals. It consists of short and medium chains of β-D-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages attached to a terminal glucose unit (Ringø *et al.* 2010). FOS supports growth and survival of bacteria present in the gastrointestinal tract, such as lactobacilli, which possess β-fructosidase to hydrolyse FOS β-(2-1) glycosidic bonds (Ringø *et al.* 2010). In fish, FOS has been assessed in red drum (*Sciaenops ocellatus*) (Buentello *et al.* 2010), sharpsnout seabream (*Diplodus puntazzo*) (Piccolo *et al.* 2011), turbot larvae (*Psetta maxima*), Atlantic salmon (*Salmo salar*), tilapia (*Oreochromis niloticus*) (Ringø *et al.* 2010), beluga (*Huso huso*) (Hoseinifar *et al.* 2011a,b) and Caspian roach (*Rutilus rutilus*) (Soleimani *et al.* 2012). In all studied species, FOS positively improved some of the analysed parameters. Short-chain fructooligosaccharides (scFOS) have the same composition as FOS, but the degree of polymerization is between $n = 1–5$; thus, for each glucose unit, there are 1 to 5 fructose units (Bornet *et al.* 2002). The fructooligosaccharide used in the present study is originated from sugar beet by enzymatic reaction and is formed by one unit of glucose and two (37%), three (53%) or four (10%) fructose units. In aquatic animals, it has only been studied to a minor extent and information is limited to hybrid tilapia (*Oreochromis niloticus*♀ × *Oreochromis aureus*♂) (Hui-Yuan *et al.* 2007; Zhou *et al.* 2009) and white shrimp (*Litopenaeus vannamei*) (Li *et al.* 2007; Zhou *et al.* 2007). In hybrid tilapia, an increase in growth (Hui-Yuan *et al.* 2007) and a modified intestinal bacterial community (Zhou *et al.* 2009) were reported when fish was fed scFOS. In white shrimp fed scFOS was reported an increase in the immunity (Li *et al.* 2007) and in growth performance (Zhou *et al.* 2007).

Studies in mammals indicate that prebiotics can affect lipid metabolism, reducing hepatic lipogenesis, concentration of liver and serum triglycerides, increase HDL/LDL ratio and have protective effect against steatosis (Delzenne *et al.* 2002, 2008; Teitelbaum 2009). However, the effect of prebiotics on key enzymes of fish lipogenesis is limited to one study in European sea bass (*Dicentrarchus labrax*) fed MOS, where the incorporation of MOS leads to a reduction on lipogenic enzyme activity (Torrecillas *et al.* 2011).

Studies on prebiotic use in turbot are limited, but some results indicate positive effects to the animals. For instance, Li *et al.* (2008) observed improved growth rate in turbot juveniles fed XOS. In turbot larvae, Mahious *et al.* (2006) evaluated inulin, oligofructose and lactosucrose as prebiotics, but only oligofructose positively affected growth.

To maximize potential benefits of prebiotics, it is essential to provide them at the most effective concentration in the diet; however, the adequate quantity can change depending on fish species, size, prebiotic type and/or rearing conditions (Merrifield *et al.* 2010). Consequently, dose-dependent studies are crucial to define the adequate level to provide the best rearing results.

Among environmental factors that affect fish metabolism, temperature is one of the most important. However, it is not known if prebiotic effects can change with seasonal cycle or temperature (Ringø *et al.* 2010). Moreover, seasonality is known to affect the resident gut microbial communities in fish, decreasing the total viable counts of bacteria at winter temperatures (Hagi *et al.* 2004).Therefore, the aim of this study was to evaluate the efficacy of scFOS in turbot juveniles reared at two temperatures, one within the range of summer water temperature and the other of winter water temperature.

Materials and methods

Diets

Four practical diets were formulated to be isolipidic (150 g kg^{-1} lipid), isoenergetic (230 kJ kg^{-1}) and isonitrogenous (550 g kg^{-1} protein). Fish meal and plant ingredients (soybean meal, wheat gluten, corn gluten, wheat meal) were used as the main protein sources (at circa 50 : 50 fish meal/plant ingredients), and fish oil was used as the lipid source (Table 1). The experimental diets included 0 g kg^{-1} (diet D0 – control diet), 5 g kg^{-1} (diet D5), 10 g kg^{-1} (diet D10) and 20 g kg^{-1} (diet D20) of scFOS (PROFEED Maxflow; Jefo, Marckolsheim, France).

All diet ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2.0 mm die. Pellets were dried in an oven (40 °C) for 48 h and stored in airtight bags until use. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Table 1 Ingredients and proximate composition of the experimental diets

	Diets			
	D0	D5	D10	D20
Ingredients (g kg^{-1} dry weight)				
Fish meal[1]	350	350	350	350
Corn gluten[2]	130	130	130	130
Wheat gluten[3]	100	100	100	100
Wheat meal[4]	17	17	17	17
Soy meal[5]	200	200	200	200
scFOS[6]	–	5	10	20
Cellulose[7]	20	15	10	–
Soluble fish protein concentrate[8]	50	50	50	50
Cod liver oil	98	98	98	98
Vitamin mix[9]	10	10	10	10
Mineral mix[10]	10	10	10	10
Choline chloride (50%)	5	5	5	5
Binder (Aquacube)[11]	10	10	10	10
Proximate analysis (g kg^{-1} dry weight)				
Dry matter	917	919	946	900
Crude protein	557	543	562	544
Crude fat	153	156	156	158
Ash	92	92	94	88
Starch	43	37	42	47
Gross energy (kJ kg^{-1})	225	230	232	225

DM, dry matter; CP, crude protein; CL, crude lipid.
[1] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 681 g kg^{-1} DM; CF: 8.8 g kg^{-1} DM).
[2] Sorgal, S.A. Ovar, Portugal (CP: 657 g kg^{-1} DM; CF: 35 g kg^{-1} DM).
[3] Sorgal, S.A. (CP: 828 g kg^{-1} DM; CF: 19 g kg^{-1} DM).
[4] Sorgal, S.A. (CP: 118 g kg^{-1} DM; CF: 19 g kg^{-1} DM).
[5] Sorgal, S.A. (CP: 532 g kg^{-1} DM; CF: 29 g kg^{-1} DM).
[6] PROFEED Maxflow 'Fructo-Oligosaccharides' (Jefo).
[7] Sigma-Aldrich, Sintra, Portugal.
[8] Sopropêche, Boulogne, France (CP: 698 g kg^{-1} DM; CF: 179 g kg^{-1} DM).
[9] Vitamins (mg kg^{-1} diet): retinol acetate, 18 000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[10] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.9; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.2; potassium iodide, 0.8; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.7; zinc oxide, 38; dibasic calcium phosphate, 5.9 (g kg^{-1} diet); potassium chloride, 1.2 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[11] Agil, London, UK (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).

Growth trial

This experiment was executed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

The experiment was performed at the experimental facilities of the Marine Zoology Station, Porto University, Portugal, with turbot (*Scophthalmus maximus*) juveniles obtained from a local commercial fish farm (Aquacria, Piscicolas, SA., Torreira, Portugal). The trial was performed in two identical recirculating water systems equipped with 12 cylindrical fibreglass tanks of 100 L water capacity and thermo-regulated to either 15.2 ± 0.7 or 20.4 ± 0.8 °C. Tanks were supplied with continuous flow of filtered seawater (2.5-3.5 L min^{-1}) with 35 ± 1 g L^{-1} salinity, and dissolved oxygen was kept near saturation using aeration (7 mg L^{-1}). After a quarantine period of 1 month, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. Before the trial fish were fed a commercial diet (500 g kg^{-1} protein and 120 g kg^{-1} lipids). Thereafter, 19 turbot, with an initial mean body weight of 31.6 ± 0.02 g, were distributed to each tank in each system and the experimental diets randomly assigned to triplicate groups within each temperature. During the trial, fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation, for 9 weeks. The utmost care was taken to avoid feed losses.

Sampling

Five fish from the initial stock population were randomly sampled and stored at -20 °C for body composition analysis. After the feeding trial, fish were starved for 24 h and sampled for biological parameters (liver and visceral indices, $n = 9$) and body composition analyses (fish pooled by tank, $n = 3$).

To minimize stress caused by manipulation, the remaining fish in each tank continued to be fed for three more days, and then, fish were randomly sampled 6 h after the morning meal for the remaining analysis (plasma metabolites, $n = 9$; liver composition analysis, $n = 9$; enzyme activity measurement, $n = 9$ from pools of three livers). Plasma after aliquot and livers for enzyme activity were stored at −80 °C and liver composition analysis at −20 °C until analysis.

Proximate analysis

Fish collected for whole-body composition were pooled by tank (thus $n = 3$) and dried in an oven at 60 °C until a constant weight was achieved. Moisture content was then calculated. Dry fish carcasses were ground for further analysis. Chemical analysis of the whole fish carcasses and diets was performed following Association of Official Analytical Chemists methods AOAC (2000). Energy content was determined

by direct combustion in an adiabatic bomb calorimeter (PARR model 1261; PARR Instruments, Moline, IL, USA) and starch according to Beutler (1984). Hepatic glycogen was determined as described by Plummer (1987), and hepatic lipid was determined by the method of Folch *et al.* (1957).

Plasma metabolites

Plasma metabolites were determined using enzymatic-colorimetric methods from Spinreact (Girona, Spain): cholesterol (kit cod. 1001090), HDL (kit cod. 1001096), LDL (kit cod. 41023), glucose (kit cod. 1001191), total lipids (kit cod. 1001270) and triglycerides (kit cod. 1001312). Plasma protein analysis was carried out using the bicinchoninic acid (BCA) Protein Assay Kit from Pierce (Rockford, IL, USA), kit cod. 23225.

Enzyme activity

Glutamate dehydrogenase (GDH, EC1.4.1.2), alanine aminotransferase (ALAT, EC2.6.1.2) and aspartate aminotransferase (ASAT, EC2.6.1.1) activities were assayed using liver extracts obtained by homogenization of frozen livers in 10 volumes (w/v) of ice-cold buffer [30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.25 mM saccharose, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM K_2HPO_4, 1 mM dithiothreitol (DTT), pH 7.4]. After centrifugation at 1000 ***g*** at 4 °C for 10 min, supernatants were sonicated for 1 min (pulse 1 s, amplitude 50) and centrifuged again at 15 000 ***g*** at 4 °C for 20 min. The resultant supernatant was separated for enzyme activity analyses. GDH activity was measured according to Bergmeyer (1974) using 10 mM of L-glutamic acid. ALAT and ASAT activities were measured using commercial kits from Spinreact: ALAT/GPT (kit ref. 41282); ASAT/GOT (kit ref. 41272).

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), fatty acid synthetase (FAS, EC 2.3.1.38) and malic enzyme (ME, EC 1.1.1.40) activities were assayed with liver extracts obtained by homogenization of frozen livers in 3 volumes (w/v) of ice-cold buffer (0.02 M Tris; 0.25 M sucrose; 2 mM EDTA; 0.1 M sodium fluoride; 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 0.01 M β-mercapto ethanol, pH 7.4), and the homogenate was centrifuged at 30 000 ***g*** at 4 °C for 20 min. G6PD activity was measured according to Bautista *et al.* (1988), FAS according to Chang *et al.* (1967) as modified by Chakrabarty & Leveille (1969) and ME activity according to Ochoa (1955).

Enzyme activity was expressed as specific activity. One unit of enzyme activity was defined as the amount of enzyme that catalysis the hydrolysis of 1 μmol min^{-1} of substrate at assay temperature (37 °C). Protein concentration was determined by the Bradford method (Bradford 1976) using Sigma protein assay kit (ref. B6916) with bovine serum albumin as a standard.

Statistical analysis

Verification for data normality and homogeneity was conducted and where necessary data were normalized (SPSS version 20 software package; IBM® SPSS® Statistics, Armonk, NY, USA). FAS and G6PD were square root normalized, and ME was logarithm normalized. All other data were not normalized. Statistical analysis of the data was conducted using a two-way ANOVA. Significant differences among means were determined using the Tukey multiple range test. The probability level for rejection of the null hypotheses was 0.05.

Results

Growth performance and feed utilization

All experimental diets were promptly accepted by the fish, and no pathological signs were observed during the trial. Mortality was very low (<4%) and not affected by diet or rearing temperature (Table 2).

Growth was higher at the higher temperature, yet thermal growth unit (TGU) was higher for fish reared at 15 °C (Table 2). There were no significant differences in final body weight and TGU between dietary treatments. However, at 15 °C, there was a positive linear correlation between prebiotic incorporation and growth (with $R = 0.986$ and $P = 0.01$). Feed intake (FI) was higher in fish reared at 20 °C than at 15 °C (Table 2), but it was not affected by dietary scFOS. Feed efficiency (FE), protein efficiency ratio (PER), nitrogen and energy retentions (NR and ER, respectively) were better in fish reared at 15 °C than at 20 °C and were not affected by dietary treatment, except for PER that was higher for fish fed the diet D20 than the diets D10 and control.

Body and liver composition

There were no differences between dietary treatments in whole-body composition, liver composition, visceral somatic indices (VSI) or hepatosomatic indices (HSI) (Table 3). Whole-body dry matter, protein, lipid and energy were also not affected by rearing temperature. How-

Table 2 Growth performance and feed utilization efficiency of turbot fed the experimental diets at two temperatures

Temperature Diets	15 °C				20 °C			
	D0	D5	D10	D20	D0	D5	D10	D20
Initial body weight (g)	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0	31.6 ± 0.0
Final body weight (g)	87.4 ± 2.0	87.9 ± 2.4	89.3 ± 2.9	90.3 ± 1.5	93.9 ± 1.3	95.8 ± 7.5	95.3 ± 2.7	95.2 ± 3.6
Thermal growth unit[1]	0.14 ± 0.00	0.14 ± 0.00	0.15 ± 0.01	0.15 ± 0.00	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.00	0.12 ± 0.00
Mortality (%)[2]	1.8 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 6.1	1.8 ± 3.0	1.8 ± 3.0	1.8 ± 3.0	0.0 ± 0.0
Feed intake (g kg ABW^{-1} per day)	11.3 ± 0.2	11.5 ± 0.1	11.9 ± 0.2	11.4 ± 0.2	12.7 ± 0.6	12.6 ± 0.5	12.6 ± 0.3	12.6 ± 0.4
Feed efficiency[3]	1.39 ± 0.02	1.36 ± 0.02	1.33 ± 0.04	1.41 ± 0.04	1.29 ± 0.04	1.32 ± 0.02	1.32 ± 0.03	1.32 ± 0.01
Protein efficiency ratio[4]	2.49 ± 0.04	2.51 ± 0.04	2.38 ± 0.07	2.59 ± 0.07	2.32 ± 0.07	2.43 ± 0.03	2.35 ± 0.06	2.44 ± 0.02
Nitrogen Retention (% NI)	36.1 ± 2.1	36.4 ± 1.6	36.4 ± 0.5	38.6 ± 0.5	33.9 ± 3.5	35.8 ± 1.0	34.6 ± 1.7	36.1 ± 1.0
Energy Retention (% EI)	34.5 ± 0.5	33.7 ± 1.2	34.2 ± 1.4	34.3 ± 1.7	31.1 ± 3.2	32.2 ± 1.9	31.5 ± 0.6	32.6 ± 0.7

Two-way ANOVA

				Diets[5]			
Variation source	Temperature	Diets	Interaction	D0	D5	D10	D20
Initial body weight (g)	ns	ns	ns	–	–	–	–
Final body weight (g)	***	ns	ns	–	–	–	–
Thermal growth unit[1]	***	ns	ns	–	–	–	–
Mortality (%)[2]	ns	ns	ns	–	–	–	–
Feed intake (g kg ABW^{-1} per day)	***	ns	ns	–	–	–	–
Feed efficiency[3]	***	ns	ns	–	–	–	–
Protein efficiency ratio[4]	***	**	ns	ab	bc	a	c
Nitrogen Retention (% NI)	*	ns	ns	–	–	–	–
Energy Retention (% EI)	**	ns	ns	–	–	–	–

ns, not significant; NI, nitrogen intake; EI, energy intake; ABW, average body weight (initial body weight + final body weight)/2.
Values presented as means ± standard deviation (±SD) (n = 3).
*P < 0.05; **P < 0.01; ***P < 0.001.
[1] Thermal growth unit (TGU): [(final body weight$^{1/3}$ − initial body weight$^{1/3}$)/(temperature × time in days)] × 100.
[2] Mortality: (number of dead fish × 100/number of initial fish).
[3] Feed efficiency (FE): (wet weight gain/dry feed intake).
[4] Protein efficiency ratio (PER): (wet weight gain/crude protein intake).
[5] Different letters in the same row stand for statistical differences between diets P < 0.05.

ever, ash levels were significantly higher in fish reared at 20 °C. VSI and HSI were higher for fish reared at 15 °C. Liver lipid (g kg^{-1}) levels were higher and liver glycogen (g kg^{-1}) levels were lower in fish reared at 20 °C.

Plasma metabolites

Plasma metabolite levels are displayed in Table 4. Plasma cholesterol, glucose and total protein were unaffected by temperature or by dietary treatment. Triglycerides, total lipids, cholesterol HDL and LDL were higher for fish reared at 20 °C, but were not affected by dietary scFOS.

Enzyme activities

Hepatic enzyme activity data are presented in Table 5. Hepatic G6PD activity was unaffected by temperature or by dietary treatment, while ME and FAS activities were higher in fish reared at 15 °C, and both enzyme activities were affected by dietary treatment. ME activity was lower in fish fed diet D20 than the control diet, and there was a trend for FAS activity to increase as dietary scFOS increased up to 10 g kg^{-1}. ASAT activity was not affected by rearing temperature or dietary treatment, while ALAT activity was not affected by dietary treatment, but was higher for fish reared at the lower temperature. GDH activity was higher for fish reared at 15 °C and for fish fed diet D0 than in fish fed diets D5 and D20.

Discussion

In the last years, several studies concerning prebiotics use in fish have arisen; however, results are somehow contradictories and some topics remained unstudied, that is, the

Table 3 Whole-body, liver composition (g kg^{-1} fresh weight), hepatosomatic and visceral indices of turbot fed the experimental diets at two temperatures

Temperature		15 °C				20 °C			
Diets	Initial	D0	D5	D10	D20	D0	D5	D10	D20
Body									
Dry matter	214 ± 0.0	214 ± 2.0	220 ± 6.7	228 ± 2.8	210 ± 8.8	216 ± 8.8	218 ± 9.4	219 ± 9.9	222 ± 6.2
Protein	133 ± 0.1	141 ± 4.0	141 ± 5.5	146 ± 2.2	143 ± 2.6	140 ± 5.1	142 ± 4.5	142 ± 3.7	143 ± 2.3
Lipid	24 ± 2.6	46 ± 1.6	43 ± 2.5	45 ± 4.4	46 ± 1.2	40 ± 5.9	44 ± 9.6	42 ± 4.0	44 ± 0.6
	42 ± 2.0	51 ± 1.0	52 ± 1.6	53 ± 1.1	50 ± 1.2	50 ± 2.2	51 ± 2.0	51 ± 1.5	51 ± 0.4
Energy (kJ kg^{-1})									
Ash	48 ± 1.6	30 ± 3.0	33 ± 0.4	34 ± 1.7	27 ± 5.5	34 ± 1.4	35 ± 3.9	35 ± 1.6	36 ± 1.1
VSI[1]	58 ± 0.9	71 ± 5.4	63 ± 4.5	60 ± 3.0	61 ± 5.3	54 ± 4.2	57 ± 6.8	58 ± 2.5	58 ± 4.6
HSI[2]	9 ± 0.3	24 ± 2.9	20 ± 3.4	20 ± 3.3	20 ± 4.0	15 ± 1.6	16 ± 3.6	16 ± 2.4	16 ± 3.4
Liver									
Lipid	–	137 ± 23	142 ± 34	145 ± 35	139 ± 43	148 ± 48	163 ± 38	167 ± 32	160 ± 58
Glycogen	–	37 ± 5.9	37 ± 7.8	38 ± 14.5	32 ± 11.0	33 ± 8.7	29 ± 5.9	30 ± 12.1	32 ± 6.3

Two-way ANOVA

				Diets			
Variation source	Temperature	Diets	Interaction	D0	D0.5	D1	D2
Body							
Dry matter	ns	ns	ns	–	–	–	–
Protein	ns	ns	ns	–	–	–	–
Lipid	ns	ns	ns	–	–	–	–
	ns	ns	ns	–	–	–	–
Energy (kJ kg^{-1})							
Ash	**	ns	ns	–	–	–	–
VSI[1]	***	ns	***	–	–	–	–
HSI[2]	***	ns	*	–	–	–	–
Liver							
Lipid	*	ns	ns	–	–	–	–
Glycogen	*	ns	ns	–	–	–	–

ns, not significant.
Values presented as means ± standard deviation (±SD) (n = 3 for body composition, n = 9 for VSI, HSI and liver composition).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[1] Visceral somatic index: (g viscera weight/kg body weight).
[2] Hepatosomatic index: (g liver weight/kg body weight).

case of influence of temperature on prebiotics effect and the effect of prebiotics in fish metabolism (Ringø *et al.* 2010; Dimitroglou *et al.* 2011).

The TGU of turbot reared at 20 °C was lower than at 15 °C. TGU is considered independent of fish weight and rearing temperature, and therefore, it is considered more adequate as parameter for comparison of growth of fish of different sizes or reared at different temperatures than other parameters, such as SGR (Bureau *et al.* 2002). At 15 °C, feed utilization efficiency was better than at 20 °C, which can be explained by this temperature being closer to the optimal temperature for growth of the species that some authors refer to be around 16 °C (Ruyet *et al.* 1991; Ruyet 2002; Danancher & Garcia-Vazquez 2007). Thus, TGU results associated with those of feed efficiency in the present study suggest that 20 °C is outside the optimal range for growth performance of turbot.

The incorporation of prebiotics, namely fructooligosaccharides, has been reported to improve growth performance of fish in several studies (Mahious *et al.* 2006; Hui-Yuan *et al.* 2007; Zhou *et al.* 2007, 2010; Soleimani *et al.* 2012), whereas other studies did not observe any growth differences (Grisdale-Helland *et al.* 2008; Buentello *et al.* 2010; Piccolo *et al.* 2011), and others even observed adverse effects (Hoseinifar *et al.* 2011a). The present data indicate no growth improvement by incorporation of scFOS in the diets of turbot juveniles. Nonetheless, the positive linear correlation found between prebiotic incorpo-

Table 4 Plasma cholesterol, HDL, LDL, glucose (mg dL^{-1}), triglycerides, total lipids and total protein (g dL^{-1}) of turbot fed the experimental diets at two temperatures

Temperature	15 °C				20 °C			
Diets	D0	D5	D10	D20	D0	D5	D10	D20
Cholesterol	103.3 ± 21.9	111.3 ± 14.3	105.2 ± 17.0	104.1 ± 4.7	115.4 ± 20.2	110.3 ± 9.3	104.4 ± 11.9	111.6 ± 10.7
Cholesterol HDL	77.9 ± 1.1	76.8 ± 10.2	58.2 ± 16.4	75.3 ± 8.7	92.1 ± 15.1	85.3 ± 15.7	84.0 ± 18.9	90.8 ± 12.9
Cholesterol LDL	59.7 ± 7.2	77.2 ± 20.3	80.1 ± 3.4	72.8 ± 6.9	95.9 ± 17.4	90.2 ± 9.2	86.2 ± 7.8	86.8 ± 8.2
Glucose	26.4 ± 8.0	28.4 ± 3.8	22.8 ± 5.9	28.9 ± 3.6	33.6 ± 3.9	32.6 ± 3.4	26.7 ± 6.8	28.8 ± 2.4
Triglycerides	0.27 ± 0.09	0.27 ± 0.08	0.30 ± 0.06	0.24 ± 0.01	0.46 ± 0.11	0.39 ± 0.03	0.36 ± 0.06	0.40 ± 0.05
Total lipids	0.93 ± 0.24	0.98 ± 0.18	0.95 ± 0.19	0.88 ± 0.03	1.59 ± 0.25	1.31 ± 0.06	1.29 ± 0.06	1.38 ± 0.16
Total Protein	4.1 ± 0.6	4.0 ± 0.5	4.3 ± 0.3	4.3 ± 0.3	4.0 ± 0.0	3.7 ± 0.2	4.1 ± 0.2	4.2 ± 0.0

Two-way ANOVA

				Diets			
Variation source	Temperature	Diets	Interaction	D0	D0.5	D1	D2
Cholesterol	ns	ns	ns	–	–	–	–
Cholesterol HDL	*	ns	ns	–	–	–	–
Cholesterol LDL	**	ns	ns	–	–	–	–
Glucose	ns	ns	ns	–	–	–	–
Triglycerides	***	ns	ns	–	–	–	–
Total lipids	***	ns	ns	–	–	–	–
Total Protein	ns	ns	ns	–	–	–	–

ns, not significant.
Values presented as means ± standard deviation (±SD) (n = 9).
*P < 0.05; **P < 0.01; ***P < 0.001.

ration and growth at 15 °C may indicate a beneficial effect of dietary prebiotic incorporation on growth performance in fish kept under optimum temperature range, and this requires further confirmation.

Feed efficiency, NR (%NI) and ER (%EI) were not affected by dietary prebiotic incorporation, at either temperature; however, PER was higher for turbot fed with 20 g kg^{-1} scFOS than the control diet. As in the present study, some previous studies observed no differences by the dietary inclusion of FOS or scFOS (Hui-Yuan *et al.* 2007; Buentello *et al.* 2010; Zhou *et al.* 2010; Piccolo *et al.* 2011), while in other studies, improved feed utilization was observed (Grisdale-Helland *et al.* 2008; Soleimani *et al.* 2012). Also in turbot, Li *et al.* (2008) observed improved feed utilization with diets including XOS. Thus, benefits of prebiotic incorporation to the diets are to date still contradictory. The efficacy of prebiotic applications is dependent on species, life stages, environmental factors and the type of prebiotic used; more studies are thus required to have a coherent picture of the effect of prebiotics in fish growth performance and feed utilization.

According to Soleimani *et al.* (2012), the better FE observed in some studies was attributable, at least partially, to an increase in digestive enzyme activities such as amylases, lipases and proteases that the authors speculated that could be related to increased exogenous microbial activity. Thus, the effect of prebiotics on microbial intestinal profile, digestive enzyme activities and nutrients digestibility in turbot should be assessed to further understand the potential effects of prebiotics.

No differences in whole-body composition were observed due to dietary scFOS supplementation. However, studies with rats reported that dietary FOS incorporation resulted in reduced fat deposition (Gibson & Roberfroid 1995). In the present study, VSI and HSI were not affected by dietary scFOS incorporation, which is in accordance with the unmodified liver lipid and glycogen levels. This also agrees with results of other studies in fish fed diets with different prebiotic supplementation (Genc *et al.* 2007; Hui-Yuan *et al.* 2007; Reza *et al.* 2009; Zhou *et al.* 2010; Hoseinifar *et al.* 2011a; Torrecillas *et al.* 2011). VSI and HSI were, however, higher in fish reared at the lower temperature. The higher HSI may be related to the higher liver glycogen content in fish reared at 15 °C and not with deposition of hepatic lipids, which were higher in fish reared at 20 °C. Although not measured, the higher VSI at 15 °C may be related to higher deposition of visceral fat at this temperature, although this higher lipid

Table 5 Specific activities (mU mg per protein) of lipogenic and amino acid catabolic enzymes in the liver of turbot fed the experimental diets at two temperatures

Temperature Diets	15 °C				20 °C			
	D0	D5	D10	D20	D0	D5	D10	D20
Lipogenesis								
Glucose 6-phosphate dehydrogenase	71.7 ± 5.1	67.6 ± 6.7	71.0 ± 13.4	62.4 ± 12.8	72.1 ± 5.2	64.2 ± 9.7	68.8 ± 8.6	70.5 ± 10.8
Malic enzyme	1.45 ± 0.34	1.18 ± 0.52	1.43 ± 0.53	1.00 ± 0.32	1.23 ± 0.41	0.99 ± 0.24	0.84 ± 0.17	0.99 ± 0.35
Fatty acid synthetase	1.7 ± 0.7	2.4 ± 0.8	2.5 ± 0.9	1.5 ± 0.5	1.1 ± 0.4	1.2 ± 0.5	1.4 ± 0.7	1.1 ± 0.3
Amino acid catabolism								
Glutamate dehydrogenase	129.3 ± 12.1	125.4 ± 10.3	132.9 ± 14.0	126.2 ± 11.2	129.5 ± 13.2	107.5 ± 9.0	116.6 ± 13.8	109.4 ± 12.1
Aspartate aminotransferase	948.6 ± 143.2	921.1 ± 79.6	966.5 ± 97.6	915.5 ± 103.2	979.2 ± 121.6	939.6 ± 63.4	914.3 ± 106.6	874.6 ± 44.1
Alanine aminotransferase	554.8 ± 60.1	516.4 ± 49.7	510.2 ± 59.5	512.1 ± 59.9	460.9 ± 38.4	444.1 ± 50.6	451.9 ± 47.2	440.2 ± 52.0

Two-way ANOVA

				Diets[1]			
Variation source	Temperature	Diets	Interaction	D0	D5	D10	D20
Glucose 6-phosphate dehydrogenase	ns	ns	ns	–	–	–	–
Malic enzyme	*	*	ns	b	ab	ab	a
Fatty acid synthetase	***	*	ns	ab	ab	b	a
Glutamate dehydrogenase	***	**	ns	b	a	ab	a
Aspartate aminotransferase	ns	ns	ns	–	–	–	–
Alanine aminotransferase	***	ns	ns	–	–	–	–

ns, not significant.
Values presented as means ± standard deviation (±SD) (n = 9).
*P < 0.05; **P < 0.01; ***P < 0.001.
[1] Different letters in the same row stand for statistical differences between diets P < 0.05.

deposition was not significantly reflected at the whole-body level.

To the authors' knowledge, there are presently no other studies available relating dietary prebiotics and the activities of amino acid metabolism enzymes in fish. In the present study, GDH was lower for fish fed 5 and 20 g kg^{-1} scFOS when compared with fish fed control diet and suggests a potential protein sparing effect by dietary scFOS. FOS incorporation on diets was reported to reduce hepatic lipogenesis in rats (Delzenne *et al.* 2002). To our knowledge, the effect of prebiotics on fish lipogenic enzymes was only previously been studied by Torrecillas *et al.* (2011) who measured ME and G6PD activities in sea bass fed diets with 2, 4 and 6 g kg^{-1} MOS. The authors concluded that MOS incorporation to the diets reduced the activity of lipogenic enzymes, and the authors suggested that it could be the result of a reduction in acetyl-CoA carboxylase activity, the rate limiting enzyme in fatty acid synthesis. However, in the present study, such reduction in lipogenesis was not apparent. On the contrary, there was a trend for FAS activity to increase as dietary scFOS increased up to 10 g kg^{-1}. However, ME activity, which provides reducing power for lipogenesis, appeared to decrease with prebiotic incorporation, being significantly lower with 20 g kg^{-1} incorporation compared to the control. More studies in fish are necessary to clarify the effect of scFOS on lipogenesis.

Dietary scFOS did not affect plasmatic levels of triglycerides, cholesterol or total lipids in the current study, which is contrary to what has been observed in mammals (Teitelbaum 2009). Oligosaccharides such as inulin and oligofructose fed to lean rats reduced plasmatic triglycerides levels mainly due to a decrease in low-density lipoproteins. In addition, it is thought that this reduction in low-density lipoproteins results in a decrease in liver triglycerides production instead of an increased catabolism of triglycerides-rich lipoproteins (Delzenne *et al.* 2002). Plasma metabolite analyses in fish fed diets with prebiotics are scarce and contradictory. Juvenile beluga fed with 10, 20 or 30 g kg^{-1} oligofructose showed no differences in plasma glucose or total protein; however, plasma cholesterol was reduced in fish fed 20 g kg^{-1} oligofructose (Hoseinifar *et al.* 2011b). On the contrary, no changes in plasma glucose, cholesterol or triglycerides were observed in beluga fed with 10, 20 or 30 g kg^{-1} inulin, but total plasma protein decreased (Reza *et al.* 2009).

It is speculated that fermentation of prebiotics by intestinal bacteria produces short-chain fatty acids such as propionate and acetate which may be related to alterations of lipogenic enzyme activities (Delzenne *et al.* 2002; Teitelbaum 2009). However, this is complicated by the fact that propionate and acetate have antagonistic effects, whereas propionate inhibits fatty acids synthesis and acetate is a lipogenic substrate. Nevertheless, in an experiment with isolated hepatocytes, the effect of propionate was stronger than that of acetate; furthermore, propionate competes with the transport protein responsible for acetate entering in the hepatocytes (Delzenne *et al.* 2002; Teitelbaum 2009). Even if acetate stimulates lipogenesis, acetate also activates AMP kinase, an enzyme related to the inhibition of lipogenesis, so lipogenesis stimulation by acetate can be somehow modulated (Delzenne *et al.* 2008).

Overall, present results indicate no benefit of scFOS incorporation to diets on growth performance of turbot, although a trend for lipogenesis increase through FAS activity increase was observed. The potential of scFOS needs to be further exploited in future studies focusing particularly on lipid metabolism, microbial intestinal profile, digestive enzyme activities and nutrients digestibility.

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Chapter 3

Effects of rearing temperature and dietary short-chain fructooligosaccharides supplementation on allochthonous gut microbiota, digestive enzymes activities and intestine health of turbot (*Scophthalmus maximus* L.) juveniles

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Effects of rearing temperature and dietary short-chain fructooligosaccharides supplementation on allochthonous gut microbiota, digestive enzymes activities and intestine health of turbot (*Scophthalmus maximus* L.) juveniles

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Abstract

The gut microbiomes of fish play important roles in host development, digestion and health. Evidence suggests that abiotic factors, such as diet and rearing temperature, could affect fish gut microbiota. In this study, the effect of dietary short-chain fructooligosaccharides (scFOS) on turbot intestinal health, microbiota and digestive enzymes was investigated at two rearing temperatures: 15 and 20 °C. Four practical diets were supplemented with scFOS at 0, 5, 10 and 20 g kg^{-1}. scFOS did not affect fish performance. PCR-DGGE did not show differences in bacterial profiles between dietary treatments; however, the number of operational taxonomic units, richness and diversity were higher at 20 °C. Enzyme activities in the foregut were not affected by rearing temperature, while in the hindgut, enzyme activities were higher at 15 °C. Total alkaline protease, α-amylase and lipase activities in the foregut were higher in fish fed 20 g kg^{-1} scFOS. Prebiotic supplementation had no effect on hindgut α-amylase activity, while lipase activity of fish reared at 20 °C was higher in fish fed diet with 5 g kg^{-1} scFOS. No differences were observed in intestinal morphology. This was the first study to simultaneously evaluate the effect of dietary prebiotic level and rearing temperature on fish intestinal microbiota and health.

KEY WORDS: digestive enzymes, gut histology, gut microbiota, rearing temperature, short-chain fructooligosaccharides, turbot

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Introduction

Prebiotics are non-digestible feed ingredients that provide substrates for the development of selected beneficial bacteria within the host gastrointestinal (GI) tract, therefore promoting GI health and immunological status (Gibson & Roberfroid 1995). In fish, prebiotics have been associated with the modulation of gut microbial communities and with the improvement of fish performance in several studies (Merrifield *et al.* 2010a; Ringø *et al.* 2010, 2014; Dimitroglou *et al.* 2011a; Daniels & Hoseinifar 2014). Fructooligosaccharides (FOS) are among the most studied and established prebiotics for fish (Merrifield *et al.* 2010a; Ringø *et al.* 2010, 2014; Dimitroglou *et al.* 2011a). While FOS comprises longer fructose units chains, in short-chain fructooligosaccharides (scFOS), the degree of polymerization is usually between 1 and 5 (Bornet *et al.* 2002). However, available data on the use of scFOS as prebiotic in fish are still scarce (Ringø *et al.* 2010, 2014). FOS is known to support growth and survival of GI tract autochthonous bacteria, such as members of the genera *Lactobacillus*, which possess ß-fructosidase activity to hydrolyse FOS ß-(2-1) glycosidic bonds. Fermentation of such compounds produces short-chain fatty acids which can be absorbed and used by enterocytes (Blaut 2002; Ringø *et al.* 2010). Moreover, modulation of GI microbiota may lead to a proliferation of gut bacteria that secrete enzymes that the host does not produce or produces in low amounts, as is the case of cellulase, phytase and chitinase (Denev *et al.* 2009; Dimitroglou *et al.* 2011a; Ray *et al.* 2012). As a consequence prebiotics have been associated with an increase in the activity of digestive enzymes in some fish species such as allogynogenetic crucian carp (*Carassius*

auratus gibelio), sleepy cod (*Oxyeleotris lineolatus*), Caspian roach (*Rutilus rutilus*), red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Xu *et al.* 2009; Renjie *et al.* 2010; Soleimani *et al.* 2012; Anguiano *et al.* 2013). Prebiotics were also reported to induce changes in morphology of fish intestine, such as increased gut absorptive area, through increased microvilli density and height (Genc *et al.* 2007; Dimitroglou *et al.* 2009, 2010, 2011b,c; Zhou *et al.* 2010). Improvements in the intestinal morphology caused by prebiotics are increasingly important as the inclusion levels of plant protein sources in aquafeeds increase. As changes in the GI microbiota, morphology of the intestine and the activity of digestive enzymes may contribute to improved growth and feed efficiency (FE), more research in this field is necessary.

Fish are heavily influenced by environmental conditions, such as water salinity, oxygen concentration and temperature. As suggested by Ringø *et al.* (2010), these factors can have an impact on fish microbiota and may influence the functional effects (efficacy) of dietary prebiotics. Among environmental factors, temperature is among the most important as fish are heterothermic animals. Gut bacteria of homoeothermic animals thrive under fairly constant temperature conditions, but in fish, due to its heterothermic nature, gut bacteria are subject to important temperature variations and this may affect resident bacterial communities (Hagi *et al.* 2004; Denev *et al.* 2009). However, the effect of water temperature on fish gut microbiota composition and on prebiotic potential in fish remains unstudied.

Previously, we have assessed in turbot (*Scophthalmus maximus*) reared at winter and summer temperatures, the effects of short-chain fructooligosaccharide (scFOS) on growth performance and hepatic intermediary metabolism (Guerreiro *et al.* 2014a) as well as hepatic oxidative status and immune response (Guerreiro *et al.* 2014b). In this work, we aimed at evaluating the effect of different levels of dietary scFOS supplementation on gut microbial community, the activity of digestive enzymes and intestinal morphology in turbot juveniles reared at two different temperatures: 15 and 20 °C. These temperatures were chosen as representative temperatures of winter and summer conditions in the Mediterranean, respectively.

Materials and methods

Diets

Four diets were formulated to be isoproteic (550 g kg^{-1} protein) and isolipidic (150 g kg^{-1} lipid) and to include fishmeal and plant ingredients (soybean meal, wheat gluten, corn gluten and wheat meal) as protein sources (50 : 50) and fish oil as the lipid source. The diets were supplemented with fructooligosaccharides (scFOS, PRO-FEED Maxflow; Jefo, Marckolsheim, France) at 5 g kg^{-1} (diet D5), 10 g kg^{-1} (diet D10) or 20 g kg^{-1} (diet D20), in exchange for cellulose. A diet with no scFOS was used as the control (diet D0). All dietary ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 2.0-mm die. Pellets were dried in an oven (40 °C) for 48 h and stored in tight bags until use.

Chemical analyses of the diets were performed following Association of Official Analytical Chemists methods (AOAC 2000). Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 1261; PARR Instruments, Moline, IL, USA) and starch according to Beutler (1984). The ingredients and proximate composition of the experimental diets are presented in Table 1.

Growth trial

This experiment was executed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

The experiment was performed at the experimental facilities of the Marine Zoology Station, Porto University, Portugal, with turbot (*S. maximus*) juveniles obtained from a local fish farm (Aquacria Piscicolas, SA., Torreira, Portugal). The trial was performed in two identical recirculating water systems equipped with 12 cylindrical fibreglass tanks of 100 L water capacity and thermoregulated to 15.2 ± 0.7 or 20.4 ± 0.8 °C. The tanks were supplied with continuous flows of filtered sea water (2.5–3.5 L min^{-1}); water salinity averaged 35 ± 1 g L^{-1} and dissolved oxygen was kept near saturation (7 mg L^{-1}). After a quarantine period of 1 month, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. During that time, fish were fed a commercial diet (500 g kg^{-1} protein and 120 g kg^{-1} lipids). Thereafter, 12 groups of 19 turbot with an initial mean body weight of 31.6 ± 0.02 g were established in each system and the experimental diets randomly assigned to triplicate groups within each temperature. The trial lasted 9 weeks, and the fish were fed by hand twice a day, until apparent visual satiation, 6 days per week.

Table 1 Ingredients and proximate composition of the experimental diets

	Diets			
	D0	D5	D10	D20
Ingredients (g kg^{-1} dry weight)				
Fishmeal[1]	350	350	350	350
Corn gluten[2]	130	130	130	130
Wheat gluten[3]	100	100	100	100
Wheat meal[4]	17	17	17	17
Soy meal[5]	200	200	200	200
scFOS[6]	–	5	10	20
Cellulose[7]	20	15	10	–
Soluble fish protein concentrate[8]	50	50	50	50
Cod liver oil	98	98	98	98
Vitamin mix[9]	10	10	10	10
Mineral mix[10]	10	10	10	10
Choline chloride (50%)	5	5	5	5
Binder (aquacube)[11]	10	10	10	10
Proximate analysis (g kg^{-1} dry weight)				
Dry matter	917	919	946	900
Crude protein	557	543	562	544
Crude fat	153	156	156	158
Ash	92	92	94	88
Starch	43	37	42	47
Gross energy (kJ kg^{-1})	225	230	232	225

scFOS, short-chain fructooligosaccharides.
[1] Steam dried LT fishmeal, Pesquera Diamante, Austral Group, S.A Perú (CP: 681 g kg^{-1} DM; CF: 88 g kg^{-1} DM).
[2] Sorgal, S.A., Ovar, Portugal (CP: 657 g kg^{-1} DM; CF: 35 g kg^{-1} DM).
[3] Sorgal, S.A., Ovar, Portugal (CP: 828 g kg^{-1} DM; CF: 19 g kg^{-1} DM).
[4] Sorgal, S.A., Ovar, Portugal (CP: 118 g kg^{-1} DM; CF: 19 g kg^{-1} DM).
[5] Sorgal, S.A., Ovar, Portugal (CP: 532 g kg^{-1} DM; CF: 29 g kg^{-1} DM).
[6] PROFEED Maxflow 'Fructo-Oligosaccharides' (Jefo).
[7] Sigma-Aldrich, Sintra, Portugal.
[8] Sopropêche, Boulogne, France (CP: 698 g kg^{-1} DM; CF: 179 g kg^{-1} DM).
[9] Vitamins (mg kg^{-1} diet): retinol acetate, 18 000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamine HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[10] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).
[11] Agil, London, UK (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).

Sampling

At the end of the trial, fish were bulk weighed after 1 day of feed deprivation. Fish continued to be fed for three more days until sampling for intestine collection. On the sampling day, fish were fed several times over the day to guarantee that intestines were full at sampling time. At 5–6 h after the first meal, seven fish per tank were randomly sacrificed with an overdose of ethylene glycol monophenyl ether. Intestines of three fish from each tank were removed, divided into two parts (foregut and hindgut), immediately frozen in liquid nitrogen and then stored at −80 °C until the measurement of digestive enzyme activities. The foregut was defined as the region after the pyloric caeca until the middle of intestine length. The hindgut was defined as the second half of the intestine until the anus.

Intestines of two other fish from each tank were sampled, and circa 1 cm of middle sections of the foregut and hindgut was collected for light microscopy analysis. The digestive contents were carefully removed, and the intestine sections rinsed in phosphate-buffered saline and fixed in marine fixative (40 ml L^{-1} formalin in marine water) for 48 h and then transferred to 700 ml L^{-1} industrial methylated spirit until histological processing.

The intestines of two other fish per tank were sampled under aseptic conditions for the collection of digesta, for the characterization of allochthonous microbiota. Digesta was obtained by squeezing the entire intestine. Samples were immediately frozen in liquid nitrogen and then stored at −80 °C until analysis.

Microbial diversity analysis

Samples of the two fish per tank were pooled to reduce variation. DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen, Crawley, UK), with a prior lysozyme incubation step at 37 °C for 30 min, and with some modifications in the DNA clean-up step, as described by Waines *et al.* (2011).

PCR amplification of the V3 region of 16 rRNA genes was conducted using 1 μL of DNA template, 25 μL of MyTaqTM Mix PCR mix (Bioline Reagents Ltd., London, UK), 22 μL of molecular biology-grade water, 1 μL of reverse primer P2 (5′-ATT ACC GCG GCT GCT GG-3′) and forward primer P1 (5′-CC TAC GGG AGG CAG CAG-3′), which had a GC clamp at 5′ end. Primers were synthesized by Eurofins MWG Biotech Ltd (Ebersberg, Germany) and used at a concentration of 50 pmol μL^{-1}. A touchdown PCR was carried out as described by Muyzer *et al.* (1993), using a Techne TC312 thermal cycler (MIDSCI, St. Louis, MO, USA), and PCR products were run on 15 g kg^{-1} agarose gel to assess PCR success in both positive and negative controls.

Denaturing gradient gel electrophoresis (DGGE) was performed using a Dcode Universal Mutation Detection System (Bio-Rad laboratories, Segrate, Italy) as described by

Merrifield *et al.* (2010b), with the exception that the PCR products were run on a 100 g kg^{-1} polyacrylamide gel at 65 V for 17 h at 60 °C. Bands of interest were excised and resuspended in 20 μL of sterile molecular biology-grade water. PCR products were cleaned using Diffinity Rapid Tip® (Sigma-Aldrich, Gillingham, UK) and sequenced by Eurofins MWG Biotech Ltd. Phylogenetic analysis to identify the closest known species was conducted by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov; Merrifield *et al.* 2010b). Minimum criteria for the acceptance were >100 bp reads and 90–100% query coverage.

Digestive enzymes activities

Each part of the intestine was homogenized (1 : 0.05 v : w) in ice-cold 50 mM Tris–HCl buffer pH 7.5. Homogenates were centrifuged at 33 000 ***g*** for 15 min at 4 °C, and the resultant supernatants were used for digestive enzyme assays.

Total alkaline protease (TAP) activity was measured according to Moyano *et al.* (1996) using tyrosine as the standard. Activity was measured based on the extinction coefficient for tyrosine (0.008 mL μg^{-1} cm^{-1}).

Lipase (EC3.1.1.3) and α-amylase (EC3.2.1.1) activities were measured using commercial kits from Spinreact (Girona, Spain; Lipase, ref. 1001275; α-Amylase, ref. 41201).

All enzyme activities were expressed as specific activity (U mg^{-1} of soluble protein for TAP and mU mg^{-1} for lipase and α-amylase). Protein concentrations were determined by the Bradford method (Bradford 1976) using a Sigma protein assay kit (ref. B6916) with bovine serum albumin (Sigma-Aldrich) as the standard.

Histological processing

Fixed samples were dehydrated in graded ethanol and embedded in paraffin wax. Then, 5 μm transverse sections were cut and stained using Haematoxylin/Eosin (H&E). The number of leucocytes infiltrated into the epithelium and the number of goblet cells across a standardized distance of 100 μm were counted in at least three different mucosal folds from each fish gut section and then averaged for all specimens in each treatment. The microscope and camera used to collect the digital images was an Olympus model VANOX-T AH-2 (Hamburg, Germany) and an OLYMPUS e-600 LS, respectively. All digital images were analysed using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data analysis was conducted using a two-way ANOVA. Data were tested for normality and homogeneity (Shapiro–Wilk and Levene tests, respectively) and when necessary, data were transformed into achieve ANOVA assumptions. When significant interaction between factors was found, a one-way ANOVA analysis was conducted for each temperature. The probability level for rejection of the null hypotheses was 0.05. Significant differences among means were determined using the Tukey multiple range test. These analyses were conducted using SPSS version 20 software (IBM® SPSS® Statistics, New York, NY, USA).

Denaturing gradient gel electrophoresis banding patterns were transformed into the presence/absence matrices for assessment between treatments using Quantity one® V4.6.3 analyses software (Bio-Rad laboratories, Hercules, CA, USA). Dice coefficients of similarity were calculated to compare the fingerprints and band intensities measured. Similarity percentages (SIMPER) were used to represent the relative similarities between treatments and replicates using Primer V6 (Clarke & Gorley 2006). Species richness was assessed using Margalef's measure of richness, and species diversity was assessed by the Shannon–Weaver index. Parameters were subjected to a two-way ANOVA, with temperature and diet as fixed factors.

Results

Growth performance

Growth performance was not the aim of this study and was presented in detail elsewhere (Guerreiro *et al.* 2014a). Briefly, during the trial, mortality was low (<2%) and not affected by diet or rearing temperature (Table 2). Growth performance, feed intake (FI) and FE (Table 2) were not affected by prebiotic incorporation. Overall, growth and FI were higher at 20 °C, although FE was higher at 15 °C.

Microbial diversity analysis

The Bray–Curtis dendrogram and V3 16S rRNA DGGE fingerprints of bacterial communities indicate that the allochthonous (Fig. 1) population had similar profiles between dietary treatments, at both temperatures, as it can be seen no clusters of dietary treatments were observed, all treatments presented high similarity percentages (>80%).

The average number of operational taxonomic units (OTUs), microbial richness, diversity and similarity

Table 2 Growth performance, feed utilization efficiency and mortality of turbot fed the experimental diets at two temperatures (Guerreiro *et al*. 2014a)

Temperature	15 °C				20 °C			
Diets	D0	D5	D10	D20	D0	D5	D10	D20
Final body weight (g)	87.4 ± 2.0	87.9 ± 2.4	89.3 ± 2.9	90.3 ± 1.5	93.9 ± 1.3	95.8 ± 7.5	95.3 ± 2.7	95.2 ± 3.6
Specific growth rate (%)[1]	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.1	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.1	1.8 ± 0.0	1.8 ± 0.1
Feed intake (g kg ABW^{-1} day^{-1})	11.3 ± 0.2	11.5 ± 0.1	11.9 ± 0.2	11.4 ± 0.2	12.7 ± 0.6	12.6 ± 0.5	12.6 ± 0.3	12.6 ± 0.4
FE[2]	1.39 ± 0.02	1.36 ± 0.02	1.33 ± 0.04	1.41 ± 0.04	1.29 ± 0.04	1.32 ± 0.02	1.32 ± 0.03	1.32 ± 0.01
Mortality (%)[3]	1.8 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 6.1	1.8 ± 3.0	1.8 ± 3.0	1.8 ± 3.0	0.0 ± 0.0

				Diets			
Variation source	Temperature	Diets	Interaction	D0	D5	D10	D20
Two-way ANOVA							
Final body weight (g)	***	ns	ns	–	–	–	–
Specific growth rate (%)[1]	***	ns	ns	–	–	–	–
Feed intake (g kg ABW^{-1} day^{-1})	***	ns	ns	–	–	–	–
FE[2]	***	ns	ns	–	–	–	–
Mortality (%)[3]	ns	ns	ns	–	–	–	–

ABW: average body weight (initial body weight + final body weight)/2; ns: not significant.
Values presented as means ± standard deviation (±SD) (n = 3).
[1] Specific growth rate (SGR): [(ln (final body weight) − ln (initial body weight))/time in days] × 100.
[2] Feed efficiency (FE): (wet weight gain/dry feed intake).
[3] Mortality: (number of dead fish × 100/number of initial fish).
*** $P < 0.001$.

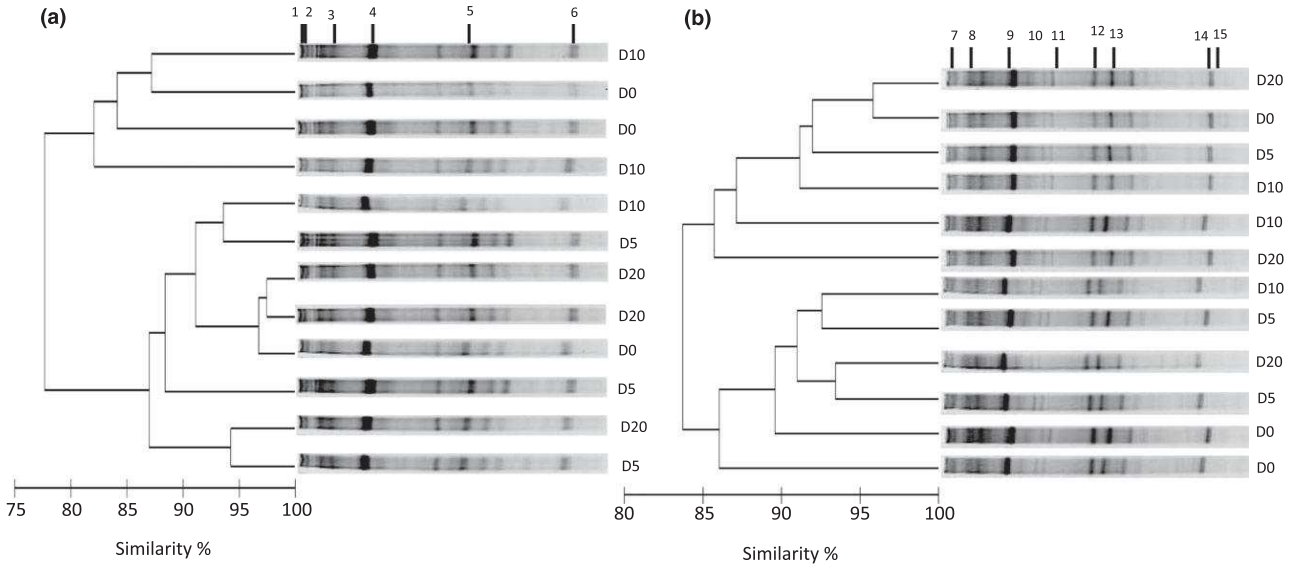

Figure 1 Dendrogram and PCR-DGGE fingerprints of the allochthonous intestinal microbiota of turbot fed the experimental diets at two rearing temperatures: 15 °C (a), 20 °C (b). Numbers (1–15) indicate bands excised for sequence analysis. Other bands were excised but failed to be sequenced.

(Table 3) were not significantly affected by prebiotic incorporation at each temperature. All parameters, except for similarity, were affected by temperature, being higher at 20 °C.

Sequence analysis from the DGGE bands (Table 4) showed that most of the dominant allochthonous bacteria detected were lactic acid bacteria (LAB) (i.e. *Weissella* and *Lactobacillus* spp.). Two OTUs were most closely related to

Table 3 Ecological parameters of PCR-DGGE fingerprints of the intestinal allochthonous microbiota of turbot fed the experimental diets at two temperatures

Temperature	15 °C				20 °C			
Diets	D0	D5	D10	D20	D0	D5	D10	D20
OTUs[1]	30.3 ± 1.2	30.3 ± 1.5	28.3 ± 2.1	31.0 ± 0.0	41.7 ± 1.5	40.7 ± 2.5	39.7 ± 3.1	38.0 ± 6.1
Richness[2]	2.8 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.8 ± 0.0	3.8 ± 0.1	3.7 ± 0.2	3.7 ± 0.2	3.5 ± 0.5
Diversity[3]	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.0	3.6 ± 0.0	3.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.2
SIMPER similarity (%)[4]	82.9 ± 2.6	86.8 ± 3.9	75.3 ± 3.6	92.1 ± 4.7	82.5 ± 2.2	86.4 ± 3.6	81.9 ± 1.2	88.0 ± 4.4

				Diets			
Variation source	Temperature	Diets	Interaction	D0	D5	D10	D20
Two-way ANOVA							
OTUs[1]	*	ns	ns	–	–	–	–
Richness[2]	**	ns	ns	–	–	–	–
Diversity[3]	***	ns	ns	–	–	–	–
SIMPER similarity (%)[4]	ns	ns	ns	–	–	–	–

ns, not significant.
Values presented as means ± standard deviation (±SD) (n = 3 per treatment pooled from six fish).
[1] OTUs: Average number of operational taxonomic units.
[2] Margalef species richness: $d = (S-1)/\log(N)$.
[3] Shannons diversity index: $H' = -\sum(\text{pi}(\ln\text{pi}))$.
[4] SIMPER, similarity percentage within group replicates.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

unidentified bacteria isolated previously either from the intestine of Atlantic cod (*Gadus morhua*) or piglets, and five OTUs were not successfully sequenced.

Digestive enzymes activities

Total alkaline protease, α-amylase and lipase activities determined in the foregut (Table 5), although not affected by rearing temperature, were significantly affected by prebiotic incorporation. TAP and lipase activities were higher in fish fed diet D20 when compared with fish fed diets D5 and D10. α-amylase activity exhibited a different behaviour at the different temperatures. In fish reared at 15 °C, α-amylase activity was significantly lower for fish fed diet D5 and at 20 °C was significantly higher in fish fed diet D20.

Total alkaline protease, α-amylase and lipase activities determined in the hindgut (Table 5) were significantly higher in fish reared at 15 °C. TAP activity was affected differently depending on the temperature. For fish reared at 15 °C, the activity was significantly higher in fish fed diet D20 when compared with fish fed diet D10; however, for fish reared at 20 °C, the activity was significantly higher when fish were fed with diets D5 and D10. Prebiotic supplementation had no effect on α-amylase activity at both temperatures. Prebiotic incorporation in the diets only affected lipase activity of fish reared at 20 °C, being significantly higher in fish fed diet D5 compared with fish fed D10 and D20.

Histological examination

Light microscopy observations showed that all experimental groups had intestinal mucosa folding similar to the control group (Fig. 2). Prebiotic incorporation in the diets did not affect intestinal epithelium gross morphology, and no signs of inflammation or intestinal damage were observed. Similarly, control and experimental groups presented an intact epithelial barrier with an organized microvilli structure. Foregut and hindgut sections contained acidophilic granulocytes and goblet cells, and the average number of epithelial leucocytes and goblet cells was not affected by dietary treatment or temperature (Table 6).

Discussion

Dietary FOS incorporation is reported to support the growth and survival of bacteria present in the GI tract of fish, such as lactobacilli (Mahious *et al.* 2006; Ringø *et al.* 2010; Hoseinifar *et al.* 2011). However, this was not observed in the present study where LAB were among the dominant bacteria identified in all diets, which might be due to the high levels of fibre and oligosaccharides present in the plant ingredients used. For example, soybean meal was incorporated in the diets at 200 g kg^{-1}, and soybean meal contains oligosaccharide contents of 40–50 g kg^{-1} which may have prebiotic-like properties, thus masking the

Table 4 Isolated bacterial bands and their closest relatives (BLAST) from PCR-DGGE of the allochthonous intestinal communities of turbot fed the experimental diets at two temperatures

Band	Rearing temperature (°C)	Groups present	Nearest neighbour	Similarity to nearest neighbour	Accession number of nearest neighbour
1	15	All groups	*Lactobacillus curvatus*	97	KC787548.1
2	15	All groups	Uncultured *Weissella* sp.	97	KC416975.1
3	15	All groups	*Lactobacillus aviarius*	100	JX986976.1
4	15	All groups	*Lactobacillus aviarius*	99	JX986976.1
5	15	All groups	*Lactobacillus crispatus*	99	KC561108.1
6	15	All groups	Uncultured bacterium from the intestine of Atlantic cod	100	HM115944.1
7	20	All groups	*Lactobacillus aviarius*	99	JX986976.1
8	20	All groups	*Lactobacillus* sp.	96	JX986976.1
9	20	All groups	*Weissella* sp.	95	KC834396.1
10	20	All groups	*Lactobacillus aviarius*	97	JX986976.1
11	20	All groups	Uncultured *Weissella* sp.	97	KC416975.1
12	20	All groups	Uncultured bacterium from ileum and caecum of weaned piglets	100	JX183818.1
13	20	All groups	*Lactobacillus helveticus*	99	HQ616643.1
14	20	All groups	*Weissella confusa*	100	KC416986.1
15	20	All groups	*Lactobacillus aviarius*	100	JX986976.1

effect of scFOS. Indeed, soybean is a natural source of oligosaccharides and is used as source of prebiotic compounds (Delzenne 2003; Gibson *et al.* 2004; Swennen *et al.* 2006). The apparent lack of prebiotic influence may have been related to the already high baseline abundance of LAB. Similarly, the gut microbiota of gilthead sea bream (*Sparus aurata*) fed a soy-rich diet was not modulated by dietary mannan oligosaccharide (MOS), but MOS did modulate the gut microbiota when supplemented to fishmeal-based diets (Dimitroglou *et al.* 2010). Previous studies have reported unchanged microbial communities in red drum fed with prebiotics (Burr *et al.* 2008b, 2009). Although PCR-DGGE is a sensitive technique, which allows identification of the dominant microbes present in environmental samples, it is at best semiquantitative. It is possible therefore that subtle changes in the abundance of OTUs might not be detected with this approach. Quantitative techniques such as FISH, qPCR and next-generation sequencing should be used in future studies as a means to overcome the semiquantitative limitations associated with PCR-DGGE (Rastogi & Sani 2011; Zhou *et al.* 2014). However, DGGE remains a useful technique which allows identification of bacteria that are often not identified using culture dependent techniques and to assess microbial community structure and ecological characteristics (Merrifield *et al.* 2010b; Zhou *et al.* 2014).

In the present study, LAB were present in turbot gut, in the form of *Lactobacillus* spp. and *Weissella* spp.; species from these genera have been reported to retard pathogenic colonization of the intestinal epithelial mucus layer, increasing fish resistance to infection, which is why they are considered as probiotic candidates (Gatesoupe 1994; Rengpipat *et al.* 2008; Mouriño *et al.* 2012; Merrifield *et al.* 2014). LAB are described as part of the regular GI microbiota of several fish species (Merrifield *et al.* 2014) but were not reported to be among the dominant components of turbot GI tract in a recent metagenomic study (Xing *et al.* 2013) which revealed Proteobacteria, particularly *Vibrio* spp., as the dominant bacteria. This discrepancy might be due to the different rearing conditions, diets or molecular microbiology techniques used.

The effect of temperature on bacterial communities of fish is somewhat scarce. In the present study, the number of allochthonous OTUs, bacterial richness and diversity were higher at 20 °C. A decrease in total viable counts of bacteria at winter temperatures has been reported in freshwater fish compared to summer temperatures (Hagi *et al.* 2004) and other studies have also reported changes in gut microbiota communities in response to water temperature (Al-Harbi & Uddin 2004; Austin 2006; Bucio *et al.* 2006). For instance, Bucio *et al.* (2006) reported that lactobacilli are more abundant at high temperatures than at low temperatures. Future studies should provide quantitative data using a variety of techniques including qPCR and next-generation sequencing libraries.

Several studies have reported increased digestive enzymatic activity or apparent nutrient digestibility in fish fed prebiotics (Burr *et al.* 2008a; Xu *et al.* 2009; Renjie *et al.* 2010; Gültepe *et al.* 2011; Soleimani *et al.* 2012). This improved digestibility may be attributed to the modulation

Table 5 Specific activities of digestive enzymes, total alkaline protease (TAP; U mg protein^{-1}), α-amylase and lipase (mU mg protein^{-1}) of foregut and hindgut intestine from turbot fed the experimental diets at two temperatures

Temperature	15 °C				20 °C			
Diets	D0	D5	D10	D20	D0	D5	D10	D20
Foregut								
TAP	46.6 ± 25.4	35.1 ± 23.3	38.8 ± 17.2	61.7 ± 15.2	54.2 ± 26.3	49.6 ± 21.4	39.6 ± 24.4	70.4 ± 29.6
α-Amylase	4.5 ± 1.3 b	2.5 ± 0.9 a	4.1 ± 1.2 b	4.5 ± 1.4 b	3.6 ± 1.1 a	3.7 ± 1.1 a	3.5 ± 1.6 a	6.2 ± 2.3 b
Lipase	0.83 ± 0.42	0.86 ± 0.62	0.84 ± 0.44	1.36 ± 0.61	1.45 ± 1.04	0.83 ± 0.70	0.54 ± 0.30	2.22 ± 1.71
Hindgut								
TAP	159.9 ± 82.5 ab	152.5 ± 72.2 ab	92.0 ± 31.8 a	232.1 ± 57.2 b	54.8 ± 25.0 a	140.5 ± 44.1 b	148.7 ± 45.4 b	40.1 ± 7.4 a
α-Amylase	4.8 ± 1.7	5.0 ± 1.0	4.0 ± 1.5	4.8 ± 1.8	3.2 ± 1.3	3.6 ± 1.0	2.4 ± 1.0	3.1 ± 1.5
Lipase	3.0 ± 1.4	3.1 ± 0.9	2.1 ± 0.9	3.5 ± 0.8	1.9 ± 1.5 ab	3.5 ± 0.7 b	1.6 ± 1.1 a	1.4 ± 0.5 a

				Diets			
Variation source	Temperature	Diets	Interaction	D0	D5	D10	D20
Two-way ANOVA							
Foregut							
TAP	ns	**	ns	ab	a	a	b
α-Amylase	ns	***	*				
Lipase	ns	**	ns	ab	a	a	b
Hindgut							
TAP	***	**	***				
α-Amylase	***	ns	ns	–	–	–	–
Lipase	**	*	*				

ns, not significant.
Values presented as means ± standard deviation (±SD) (n = 9). Means with different letters indicate significant differences among means ($P < 0.05$).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

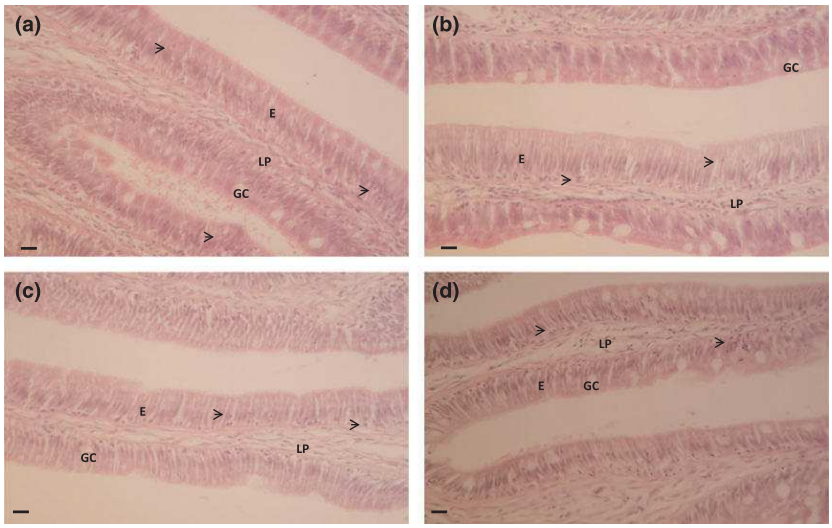

Figure 2 Histology haematoxylin/eosin (H&E) staining of the foregut and hindgut of turbot fed the experimental diets at two temperatures. (a) Foregut of fish fed diet D0 reared at 20 °C; (b) Hindgut of fish fed diet D5 reared at 15 °C; (c) Hindgut of fish fed diet D10 reared at 20 °C; (d) Hindgut of fish fed diet D20 reared at 20 °C. Scale bars represent 10 μm. Abbreviations used are GC, goblet cells; E, epithelium; LP, lamina propria. Arrows point to leucocytes infiltrated into the epithelium.

Table 6 Number of leucocytes and goblet cells residing between every 100 μm in the foregut and hindgut of turbot fed the experimental diets at two temperatures

Temperature	15 °C				20 °C			
Diets	D0	D5	D10	D20	D0	D5	D10	D20
Foregut								
Leucocytes	16.2 ± 5.2	23.9 ± 9.5	17.9 ± 2.2	17.0 ± 3.7	15.9 ± 5.5	15.8 ± 3.4	18.6 ± 5.5	20.3 ± 6.4
Goblet cells	2.3 ± 1.4	3.5 ± 2.4	2.2 ± 1.3	4.6 ± 2.0	2.8 ± 2.2	1.4 ± 1.2	2.3 ± 0.4	2.5 ± 2.2
Hindgut								
Leucocytes	19.0 ± 6.1	16.6 ± 2.3	14.2 ± 2.3	14.3 ± 4.5	18.9 ± 4.7	16.7 ± 4.9	18.3 ± 5.2	18.1 ± 4.6
Goblet cells	1.9 ± 1.0	3.1 ± 1.7	2.0 ± 1.3	2.3 ± 1.4	2.4 ± 1.0	2.9 ± 1.3	3.1 ± 1.2	2.7 ± 2.4

Values presented as means ± standard deviation (±SD) (n = 6).
No significant differences were noticed between dietary treatments, temperature or number of leucocytes and goblet cells (two-way ANOVA: $P > 0.05$).

of gut microbiota and/or consequent increased production of several bacterial enzymes, such as cellulase, chitinase, amylase, protease, lipase, phytase, tannase or xylanase, to name a few (Bairagi *et al.* 2002; Ray *et al.* 2012). In this study, digestive enzymatic activity in the hindgut was higher in fish reared at 15 °C. Although diet digestibility was not evaluated, these observations indicate higher diet digestibility which correlates well with the improved FE observed in fish reared at 15 °C. Interestingly, fish reared at 15 °C had a significantly lower FI compared to fish reared at 20 °C (Guerreiro *et al.* 2014a). However, despite the better FE, growth at 15 °C was significantly lower than in fish reared at 20 °C. In the present study, a higher digestive enzyme activity was noticed at the lower temperature, similarly to what was observed in other fish species, namely gilthead sea bream, yellowtail kingfish (*Seriola lalandi*) and yellowtail (*Seriola quinqueradiata*) (Kofuji *et al.* 2005; Miegel *et al.* 2010; Couto *et al.* 2012; Sánchez-Muros *et al.* 2013). The higher enzymatic activity measured in fish reared at lower temperatures may be related with a slower gut transit time that allows an accumulation of enzymes in the digesta, therefore increasing the time during which enzymes may exert their digestive functions (Kofuji *et al.* 2005; Miegel *et al.* 2010). Other possible explanation may be related to higher enzymatic secretion to maximize the nutrient digestion and absorption of the lower amount of fed ingested at lower temperatures (Miegel *et al.* 2010).

Although a relation between prebiotic incorporation, digestive enzymes activity and gut bacterial population can be assumed, in the current study, it was not possible to establish a correlation between dietary scFOS supplementation, microbiota population and enzyme activity as was observed in poultry (Xu *et al.* 2003). Previous studies with fish and crustaceans have reported increased digestive enzymes activities in fish fed prebiotic supplemented diets (Xu *et al.* 2009; Renjie *et al.* 2010; Sang *et al.* 2011). In the

present study, however, incorporation of scFOS in the diets did not improve the digestive enzymes activity except for fish fed 20 g kg^{-1} scFOS that presented higher absolute values of enzymatic activity in the foregut, although being higher, the values are not significantly higher than the ones registered in fish fed control diet. This is contrary to the results of Soleimani *et al.* (2012), who observed increased amylase, lipase and protease activities in Caspian roach fry fed increasing FOS levels (10, 20 and 30 g kg^{-1}).

Some studies reported increased absorptive area due to increased microvilli density and height in fish fed prebiotics (Genc *et al.* 2007; Dimitroglou *et al.* 2009, 2010, 2011b,c; Zhou *et al.* 2010). However, in the present study, no differences in intestinal morphology were observed among dietary treatments. The current study did not assess the intestinal ultrastructure, and it is possible that at this level changes may have occurred, as has been observed in juvenile trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*) fed MOS (Torrecillas *et al.* 2007; Dimitroglou *et al.* 2009). Future studies are required to assess the impact of FOS on the intestinal ultrastructure of fish.

In conclusion, fish reared at 15 °C presented higher enzymatic activity, possibly supporting better digestive capacity in fish reared under this temperature. However, the present results did not detect an appreciable effect of scFOS incorporation in turbot juvenile's digestive enzymes, intestinal bacterial richness or diversity. Nonetheless, fish reared at 20 °C had higher intestinal bacterial richness and diversity. Future studies should incorporate more sensitive techniques to elucidate bacterial abundance and activity.

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Chapter 4
Effect of temperature and short chain fructooligosaccharides supplementation on the hepatic oxidative status and immune response of turbot (*Scophthalmus maximus*)

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Effect of temperature and short chain fructooligosaccharides supplementation on the hepatic oxidative status and immune response of turbot (*Scophthalmus maximus*)

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ABSTRACT

In this study the effect of diet supplementation with different levels of short-chain fructooligosaccharides (scFOS) on the hepatic oxidative status, hematology and innate immune parameters was evaluated in turbot reared at 15 °C and 20 °C. Four practical diets containing half of the protein provided by plant ingredients and the other half by fish meal were supplemented with scFOS at 0%, 0.5%, 1.0% and 2.0% and fed to turbot juveniles for 9 weeks. Independently of the rearing temperature, diet with 1% scFOS increased the haematocrit (Ht) while 2% scFOS augmented the mean corpuscular haemoglobin concentration (MCHC). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), white blood cells (%) and lysozyme were higher in fish reared at 15 °C, whereas red blood cells and neutrophil numbers increased in fish reared at 20 °C. Catalase (CAT) and glutathione peroxidase (GPX) activities were affected by rearing temperature being lower in fish reared at 20 °C. Compared to the control diet, at 15 °C, turbot fed 0.5 or 1% scFOS presented lower activities of CAT and glutathione reductase (GR). At 20 °C turbot fed the 2% scFOS diet presented lower activities of CAT and GPX. Lipid peroxidation (LPO) and glucose 6-phosphate dehydrogenase (G6PDH) activity were not affected by temperature nor dietary prebiotic incorporation. Results of this study suggest scFOS has no effect on innate immunology or hematology. High temperature (20 °C) does not induce turbot oxidative stress, but the recommended dietary scFOS incorporation level for counteracting oxidative stress may differ with other rearing temperature.

1. Introduction

Prebiotics are defined as non-digestible feed ingredients, providing substrates for the development of selected beneficial bacteria within the host gastrointestinal tract, therefore promoting gastrointestinal health and immunological status [1]. Prebiotics may therefore be used for the immunoprophylactic control of fish diseases, as a way of improving both growth efficiency and the immune system [2]. Moreover, prebiotics such as inulin were reported as antioxidants, with the ability of ROS scavenging [3,4].

Fructooligosaccharides (FOS) is a common prebiotic used in humans and farm animals, and has already been studied in a few fish species, although studies in fish were mostly limited to growth performance, feed efficiency and immune responses [5–9]. Studies accessing the effect of FOS on fish oxidative status are scarce. Ref. [10] studied the effect of FOS on triangular bream (*Megalobrama terminalis*) and observed immune modulation and enhanced antioxidant capability. Ref. [8] observed increased serum superoxide dismutase (SOD) activity in large yellow croaker (*Larimichthys crocea*) juveniles fed a combination of *Bacillus subtilis* and FOS, whereas Ref. [11] observed increased serum SOD activity in turbot (*Scophthalmus maximus*) juveniles fed with a combination of xylooligosaccharides (XOS), yeast cell wall, and bile acids.

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Turbot is a high market value aquaculture species with an estimated production in 2012 of 77,000 tons [12]. Studies on prebiotic use in turbot feeds are scarce. As mentioned above Ref. [11] evaluated the effect of XOS, yeast cell wall and bile acid on nonspecific immunity and growth of turbot juveniles and Ref. [5] studied the effect of inulin, oligofructose and lactosucrose on turbot weaning. Recently, Ref. [13] studied the effect of short-chain fructooligosaccharides (scFOS) on growth performance and hepatic intermediary metabolism of turbot reared at winter and summer temperatures. scFOS has the same molecular composition as FOS but a shorter degree of polymerisation, usually between 1 and 5 fructose units for each glucose unit [14]. Results of Ref. [13] seem to indicate no benefit on growth performance of incorporating scFOS to diets of turbot reared at winter and summer temperature, though a trend for lipogenesis increase through fatty acid synthetase activity increase was observed.

While recent studies reported that water temperature may influence antioxidant defenses and non-specific immunity of fish [2,15,16], it is still not known whether dietary prebiotic effects can be influenced by seasonal cycle or water temperature [17]. Therefore, the aim of the present study was to assess the effect of different dietary levels of scFOS supplementation on the hepatic oxidative status, hematology and immune responses of turbot reared at two temperatures: 15 °C, which is within the optimal rearing temperature for turbot juveniles growth [18–20], and 20 °C, which is within the range of upper temperatures for turbot rearing.

2. Material and methods

2.1. Diets

Four practical diets were formulated to be isonitrogenous (55% protein) and isolipidic (15% lipid). Fish meal (FM) and plant feedstuffs (PF: soybean meal, wheat gluten, corn gluten, wheat meal) were used as the main protein sources (circa 50 FM:50 PF) and fish oil was used as lipid source. The experimental diets included 0% (diet D0; control diet); 0.5% (diet D0.5); 1.0% (diet D1) and 2.0% (diet D2) of scFOS. All diet ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2.0 mm die. Pellets were dried in an oven (40 °C) during 48 h and stored in tight bags at room temperature until used. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Table 1
Ingredients and proximate composition of the experimental diets.

	Diets			
	D0	D0.5	D1	D2
Ingredients (dry weight basis)				
Fish meal[a]	35.0	35.0	35.0	35.0
Corn gluten[b]	13.0	13.0	13.0	13.0
Wheat gluten[c]	10.0	10.0	10.0	10.0
Wheat meal[d]	1.7	1.7	1.7	1.7
Soy meal[e]	20.0	20.0	20.0	20.0
scFOS[f]	–	0.5	1.0	2.0
Cellulose[g]	2.0	1.5	1.0	–
Soluble fish protein concentrate[h]	5.0	5.0	5.0	5.0
Cod liver oil	9.8	9.8	9.8	9.8
Vitamin mix[i]	1.0	1.0	1.0	1.0
Mineral mix[j]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder (aquacube)[k]	1.0	1.0	1.0	1.0
Proximate analysis (% dry weight)				
Dry matter (%)	91.7	91.9	94.6	90.0
Crude protein	55.7	54.3	56.2	54.4
Crude fat	15.3	15.6	15.6	15.8
Ash	9.2	9.2	9.4	8.8
Starch	4.3	3.7	4.2	4.7
Gross energy (kJ g^{-1})	22.5	23.0	23.2	22.5

DM: dry matter; CP: crude protein; CF: crude fat.
[a] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 68.1% DM; CF: 8.8% DM).
[b] Sorgal, S.A. Ovar, Portugal (CP: 65.7% DM; CF: 3.5% DM).
[c] Sorgal, S.A. Ovar, Portugal (CP: 82.8% DM; CF: 1.9% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 11.8% DM; CF: 1.9% DM).
[e] Sorgal, S.A. Ovar, Portugal (CP: 53.2% DM; CF: 2.9% DM).
[f] PROFEED Maxflow "Fructo-Oligosaccharides" (Jefo, France).
[g] Sigma-Aldrich, Sintra, Portugal.
[h] Sopropêche, France (CP: 69.8% DM; CF: 17.9% DM).
[i] Vitamins (mg kg^{-1} diet): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[j] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).
[k] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).

2.2. Growth trial

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with turbot (*Scophthalmus maximus*) juveniles obtained from a local fish farm (Aquacria Piscicolas S.A., Torreira, Portugal). The trial was performed in 2 identical recirculating water systems equipped with 12 cylindrical fiberglass tanks of 100 L water capacity and thermo-regulated to 15.2 ± 0.7 °C and 20.4 ± 0.8 °C. Tanks were supplied with continuous flow of filtered seawater (2.5–3.5 L min^{-1}), water salinity averaged 35 ± 1 g L^{-1} and dissolved oxygen was kept near saturation. After a quarantine period of one month, fish were transferred to the experimental systems and adapted to the rearing conditions for 15 days. Thereafter, 12 groups of 19 turbot with an initial mean body weight of 31.6 ± 0.02 g were established in each system. The experimental diets were randomly assigned to triplicate groups within each temperature and the trial lasted 9 weeks. During that period fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation.

This experiment was executed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

2.3. Sampling

At the end of the trial 3 fish per tank were randomly sampled 6 h after the morning meal and anesthetized with phenoxyethanol (0.3 mL L^{-1}). Blood was then collected from the caudal vein using heparinized syringes and placed in heparinized tubes. One aliquot was used for hematological assessment while the remaining blood was centrifuged at 3000 × *g* for 10 min at room temperature. The resulting plasma was frozen at −80 °C until analysis. After blood collection livers were removed, immediately frozen in liquid nitrogen, and then stored at −80 °C for enzyme activity measurement.

2.4. Proximate analysis

Chemical analyses of the diets were performed following Association of Official Analytical Chemists methods [21]. Energy content was determined by direct combustion in an adiabatic bomb

calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA) and starch analysis according to [22].

2.5. Hematological analysis

Fresh heparinized blood was used for hematocrit (Ht) and hemoglobin (Hb) determination and blood cells counts. Ht value was determined by microcentrifugation (10,000 × *g* for 10 min, at room temperature); Hb was determined using the Drabkin's solution (Spinreact, ref. 1001230; Girona, Spain). Total red blood cells (RBC) and white blood cells (WBC) were counted from blood dilutions using a hemocytometer. Blood smears were prepared from fresh blood, air dried, and stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol–ethanol (10% of 37% formaldehyde in absolute ethanol). Detection of peroxidase activity to label neutrophils was done according to [23]. The slides were examined under oil immersion (1000×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The percentage of each leucocyte type was then calculated.

2.6. Lysozyme activity

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ref. [24]. Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} 0.05 M sodium phosphate buffer, pH 6.2) was prepared. Then, 15 μL of plasma and 250 μL of the above suspension were added to a microplate. The reaction was carried out at 25 °C and the absorbance read at 450 nm after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to develop a standard curve. The amount of lysozyme in samples was calculated based on the standard curve.

2.7. Alternative complement pathway (ACP) activity

ACP was estimated as described by Ref. [25]. The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA–GVB, as the previous one but containing 20 mM EDTA; Mg–EGTA–GVB, which is GVB with 10 mM Mg^{2+} and 10 mM EDTA. Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. Then, 10 μL of RaRBC suspension was added to 40 μL of serially diluted plasma in Mg–EDTA–GVB buffer. Samples were incubated at room temperature for 100 min with occasional shaking. The reaction was stopped by adding 150 μL of cold EDTA–GVB. Samples were then centrifuged and the extent of hemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of plasma giving 50% hemolysis of RaRBC.

2.8. Total peroxidase activity

Total peroxidase activity was measured according to [26]. Shortly, 15 μL of plasma were diluted with 135 μL of HBSS (Hank's Balanced Salt Solution) without Ca^{+2} and Mg^{+2}. Thereafter, 50 μL of 10 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 μL of 5 mM H_2O_2 were added. The color-change reaction was stopped after 2 min by adding 50 μL of 2 M sulphuric acid and the absorbance read at 450 nm. Wells without plasma were used as blanks. The peroxidase activity (units per mL^{-1} of plasma) was then determined. One unit of peroxidase was defined as 1 OD change in absorbance.

2.9. Total immunoglobulin

Plasma total immunoglobulin (Ig) was determined according to [27]. The assay was based on the measurement of total protein content in plasma using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, ref. 23225; Rockford, USA) prior to and after precipitating the immunoglobulin molecules employing a 12% solution of polyethyleneglycol (Sigma). The difference in protein content is considered to represent total plasma immunoglobulin content.

2.10. Oxidative enzymes activity

The liver samples used for oxidative enzyme assays were homogenized in five volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. The procedure was performed on ice. Homogenates were centrifuged at 30,000 × *g* for 30 min at 4 °C and the resultant supernatants were separated in aliquots and stored at −80 °C until use. All enzyme assays were carried out at 25 °C using a microplate reader (ELx808; Bio-Tek Instruments). The optimal substrate and protein concentrations for measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 mM^{-1} cm^{-1}, respectively.

Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione reductase (GR; EC 1.6.4.2) were determined as previously described by Ref. [28].

Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was assayed with liver extracts obtained by homogenization of frozen livers in 3 volumes (w/v) of ice cold buffer (0.02 M Tris; 0.25 M sucrose; 2 mM EDTA; 0.1 M sodium fluoride; 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 0.01 M β-mercapto ethanol, pH 7.4) and the homogenates were centrifuged at 30,000 × *g* at 4 °C for 20 min. Activity was measured according to [29].

Protein concentration in homogenates was determined by the Bradford method [30] using Sigma protein assay kit (ref. B6916) with bovine serum albumin as a standard.

Enzyme activity was expressed as units (SOD, CAT) or milliunits (GPX, GR and G6PDH) per mg of hepatic soluble protein. Except for SOD, one unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the assay conditions. One unit of SOD activity was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome *c* reduction rate.

2.11. Lipid peroxidation (LPO)

The concentration of malondialdehyde (MDA) was used as marker of LPO levels in the liver. In the presence of thiobarbituric acid, MDA reacts producing colored thiobarbituric acid reacting substances (TBARS) that were measured as previously described by Ref. [28]. Results were expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

2.12. Statistical analysis

Data analysis was done by two-way ANOVA using the SPSS version 20 software package (IBM® SPSS® Statistics, New York, USA). Data was tested for normality and homogeneity of variances (Shapiro–Wilk and Levene tests, respectively) and when necessary data was normalized (arcsine, square root or logarithm normalized) to achieve ANOVA assumptions. When significant interactions between factors were found, one-way ANOVA analysis was done within each temperature. The probability level for rejection of the

null hypotheses was 0.05. Significant differences among means were determined using the Tukey multiple range test.

3. Results

3.1. Growth performance

Results of the feeding trial were presented elsewhere [13]. Overall, during the trial mortality was low (<4%) and not affected by diet or rearing temperature. Growth performance and feed intake were higher in fish reared at the higher temperature and were not affected by dietary prebiotic incorporation.

3.2. Hematological parameters

Mean corpuscular hemoglobin concentration (MCHC) and Ht were affected by dietary scFOS incorporation but not rearing temperature. MCHC was higher in fish fed diet D2 than the other diets, whereas Ht was lower in fish fed diet D2 than diet D1. On the contrary, RBC (both absolute numbers and percentage), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and WBC (%) were affected by rearing temperature but not by diet composition. Fish reared at 20 °C had higher RBC and lower MCV, MCH and WBC (%) than fish reared at 15 °C (Table 2). Except for neutrophils, white blood cells were not affect by diet or temperature (Table 3). Neutrophils, both in absolute numbers and as proportion of leucocytes, were affected by rearing temperature, being higher in fish reared at 20 °C.

3.3. Immune parameters

Peroxidase, ACP activity and total Ig levels were not affected by dietary treatments, whereas lysozyme activity was higher in fish reared at 15 °C (Table 4).

3.4. Antioxidant enzymes and LPO

Antioxidant enzymatic activity and LPO levels are present in Table 5. G6PDH activity and LPO levels were not affected by rearing temperature nor dietary treatment. CAT and GPX were the only enzymes affected by rearing temperature, with activities being lower in fish reared at 20 °C. Except for G6PDH, there was a significant interaction between diets and temperature in the activities of all enzymes. SOD and GR activities were only affected by dietary treatment. At 15 °C SOD activity was lower in fish fed diet D1 than the control diet, while at 20 °C SOD activity was higher in fish fed diets D1 and D2 than the control diet. GR activity was not affected by dietary treatment in fish reared at 20 °C while at 15 °C it was higher in fish fed the control diet than diets D0.5 and D1. Comparatively to the control, CAT activity tended to be lower in fish fed the experimental diets. GPX activity was highly variable in relation to temperature and diet composition. At 15 °C GPX activity was lower in fish fed diet D0.5 than diets D1 and D2. On the contrary, at 20 °C GPX activity was higher in fish fed diet D0.5 than the control diet.

4. Discussion

Hematological parameters are valuable tools for assessing the physiological and health status of animals as blood parameters are considerably resistant to alterations being for that reason good indicators when examining the effects of immunostimulants [31]. Results of different studies are however difficult to compare as differences observed may be related to the complexity of prebiotic tested and variables such as rearing conditions, fish species and fish size, or sampling procedures [31]. In the present study hematological parameters were barely affected by diet composition; only Ht and MCHC were affected by dietary scFOS. Similar to the present study, gilthead seabream fed 0.2 and 0.4% mannanoligosaccharides

Table 2
Red (RBC) and white (WBC) blood cells counts, hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of turbot fed the experimental diets at two temperatures.

Temperature	15 °C				20 °C			
Diets	D0	D0.5	D1	D2	D0	D0.5	D1	D2
RBC ($\times 10^6$ mm^{-3})	1.3 ± 0.2	1.3 ± 0.3	1.4 ± 0.5	1.3 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	1.6 ± 0.3
RBC (%)	98.8 ± 1.3	96.5 ± 1.2	96.5 ± 1.1	96.5 ± 1.0	97.3 ± 0.6	97.0 ± 0.6	97.2 ± 0.4	97.3 ± 0.7
Hematocrit (%)	16.7 ± 2.1	17.9 ± 2.3	19.1 ± 1.9	15.8 ± 1.8	18.2 ± 2.2	19.0 ± 2.3	19.3 ± 3.2	17.1 ± 2.9
Hemoglobin (g dl^{-1})	4.1 ± 1.0	3.8 ± 0.6	4.1 ± 0.4	4.2 ± 0.3	3.9 ± 0.6	4.5 ± 1.0	4.7 ± 1.1	4.7 ± 1.0
MCV (μm^3)[a]	130.4 ± 10.0	140.8 ± 27.7	141.2 ± 26.9	120.8 ± 7.8	107.4 ± 18.7	126.7 ± 12.7	117.0 ± 16.9	109.7 ± 33.6
MCH (pg cell^{-1})[b]	32.5 ± 9.1	29.6 ± 5.1	30.5 ± 7.4	32.8 ± 5.0	22.6 ± 3.2	28.2 ± 2.6	28.8 ± 8.5	30.3 ± 10.3
MCHC (g 100 mL^{-1})[c]	24.7 ± 5.5	21.1 ± 1.3	21.4 ± 1.7	27.3 ± 4.9	21.6 ± 4.8	22.3 ± 1.5	24.3 ± 5.0	27.6 ± 3.9
WBC ($\times 10^4$ mm^{-3})	4.1 ± 1.2	4.6 ± 1.0	4.9 ± 1.0	4.9 ± 1.1	4.7 ± 0.9	4.6 ± 0.9	4.8 ± 1.0	4.5 ± 1.0
WBC (%)	3.2 ± 1.3	3.5 ± 1.2	3.5 ± 1.1	3.6 ± 0.7	2.7 ± 0.6	3.0 ± 0.6	2.8 ± 0.4	2.7 ± 0.7

Two-way ANOVA

Variation source	Temperature	Diets	Interaction	Diets			
				D0	D0.5	D1	D2
RBC ($\times 10^6$ mm^{-3})	***	ns	ns	–	–	–	–
RBC (%)	*	ns	ns	–	–	–	–
Hematocrit (%)	ns	*	ns	ab	ab	b	a
Hemoglobin (g dl^{-1})	ns	ns	ns	–	–	–	–
MCV (μm^3)[a]	**	ns	ns	–	–	–	–
MCH (pg cell^{-1})[b]	*	ns	ns	–	–	–	–
MCHC (g 100 mL^{-1})[c]	ns	**	ns	a	a	a	b
WBC ($\times 10^4$ mm^{-3})	ns	ns	ns	–	–	–	–
WBC (%)	*	ns	ns	–	–	–	–

Values presented as means ± standard deviation (±SD) ($n = 6$).
Two-way ANOVA: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns: not significant. Different letters stand for significant differences between diets.
[a] MCV (mean corpuscular volume): (Hematocrit/10^6 μL^{-1} erythrocytes) × 10.
[b] MCH (mean corpuscular hemoglobin): (Hemoglobin/10^6 μL^{-1} erythrocytes) × 10.
[c] MCHC (mean corpuscular hemoglobin concentration): (Hemoglobin/Hematocrit) × 100.

Table 3
Differential white blood cell counts: absolute numbers and proportion of thrombocytes, lymphocytes, monocytes and neutrophils of turbot fed the experimental diets at two temperatures.

Temperature	15 °C				20 °C			
Diets	D0	D0.5	D1	D2	D0	D0.5	D1	D2
Thrombocytes ($\times 10^4$ mm^{-3})	1.28 ± 0.45	1.21 ± 0.42	1.37 ± 0.32	1.14 ± 0.34	0.97 ± 0.43	1.68 ± 0.83	1.24 ± 0.25	1.07 ± 0.40
Thrombocytes (% WBC)	32.2 ± 12.1	26.4 ± 7.7	28.5 ± 7.4	23.6 ± 5.7	20.4 ± 8.2	36.8 ± 16.3	26.6 ± 7.0	23.9 ± 8.2
Lymphocytes ($\times 10^4$ mm^{-3})	2.60 ± 1.10	3.05 ± 0.73	3.32 ± 0.95	3.61 ± 0.90	3.36 ± 0.72	2.58 ± 0.96	3.19 ± 0.81	3.05 ± 0.86
Lymphocytes (% WBC)	62.8 ± 11.2	66.6 ± 6.0	66.6 ± 7.8	73.3 ± 5.1	71.7 ± 8.9	56.5 ± 18.6	65.9 ± 4.3	67.8 ± 6.4
Monocytes ($\times 10^4$ mm^{-3})	0.08 ± 0.08	0.12 ± 0.09	0.08 ± 0.05	0.06 ± 0.03	0.06 ± 0.10	0.08 ± 0.04	0.09 ± 0.03	0.08 ± 0.05
Monocytes (% WBC)	1.87 ± 1.95	2.60 ± 1.68	1.81 ± 1.26	1.35 ± 0.80	1.44 ± 2.62	1.82 ± 1.02	1.78 ± 0.48	1.93 ± 0.90
Neutrophils ($\times 10^4$ mm^{-3})	0.11 ± 0.04	0.20 ± 0.19	0.15 ± 0.03	0.09 ± 0.07	0.29 ± 0.19	0.23 ± 0.17	0.29 ± 0.18	0.26 ± 0.12
Neutrophils (% WBC)	3.08 ± 1.89	4.40 ± 3.65	3.12 ± 1.12	1.76 ± 1.12	6.47 ± 5.05	4.89 ± 2.82	5.73 ± 3.17	6.30 ± 3.97

Two-way ANOVA

Variation source	Temperature	Diets	Interaction
Thrombocytes ($\times 10^4$ mm^{-3})	ns	ns	ns
Thrombocytes (% WBC)	ns	ns	ns
Lymphocytes ($\times 10^4$ mm^{-3})	ns	ns	ns
Lymphocytes (% WBC)	ns	ns	ns
Monocytes ($\times 10^4$ mm^{-3})	ns	ns	ns
Monocytes (% WBC)	ns	ns	ns
Neutrophils ($\times 10^4$ mm^{-3})	***	ns	ns
Neutrophils (% WBC)	***	ns	ns

Values presented as means ± standard deviation (±SD) ($n = 6$).
Two-way ANOVA: ***$P < 0.001$; ns: not significant.

presented no differences in the hematological parameters analyzed [32]. On the other hand, Ref. [33] reported higher Hb levels in beluga (*Huso huso*) juveniles fed 1 and 2% oligofructose whereas individuals fed 2% oligofructose presented an increase in Ht and leucocyte counts. That study further suggests that 3% dietary oligofructose may have adverse effects on some hematological parameters and that the elevated leucocyte numbers observed may represent an immunomodulatory effect. On the contrary, Ref. [34] observed decreased leucocyte counts, Hb, Ht, and MCV in beluga fed 2 and 3% inulin while MCHC increased in fish fed 3% inulin.

In this study MCV, MCH, RBC, WBC, and neutrophil counts were affected by rearing temperature. Fish reared at 20 °C showed increased RBC and neutrophil numbers relatively to fish reared at 15 °C. Since fish O_2 demand increases, while O_2 dissolved in water decreases, with temperature increase it was to expect an increase of RBC number, as erythrocytes are responsible for carrying O_2. Such response was already reported for rainbow trout (*Oncorhynchus mykiss*) exposed to simulated winter, spring and summer conditions [35]. These authors also reported an increased neutrophil number and improved phagocytic ability in fish reared at summer temperature. As in that study, in the present work fish reared at lower temperature also presented higher MCV and MCH values. Similar to the present data, Ref. [36] also did not observe any differences in Ht in turbot reared at 11, 14, 17 and 20 °C.

Dietary prebiotics may contribute to improving fish innate and adaptive immune responses [2,37]. For instance, in red drum (*Sciaenops ocellatus*) lysozyme activity increased in fish fed diets supplemented with FOS [38]. An increase in the Ig levels, lysozyme activity, and ACP was also reported in Caspian roach (*Rutilus rutilus*) fry fed 2 and 3% FOS [39]. However, in the present study no effect of scFOS dietary inclusion was observed in the immune parameters assessed. Similarly, Ref. [6] also did not observe differences in the immune parameters of Atlantic salmon (*Salmo salar*) fed FOS, while in triangular bream only immunoglobulin *M* was increased after feeding FOS [10]. It is possible that FOS does not exert immunological effects since to current knowledge there are no specific cellular FOS receptors on vertebrate cells [17]. Nevertheless, it cannot be discarded that the lack of immune response due to scFOS supplementation can be related to the duration of administration. Indeed, Ref. [40] observed increased neutrophil numbers after 2 weeks of feeding African catfish (*Clarias gariepinus*) with Macro-Gard® (source of beta-1,3/1,6-glucans), whereas after 45 days those

Table 4
Lysozyme, total immunoglobulin (Ig), peroxidase and alternative complement pathway activity (ACP) of turbot fed the experimental diets at two temperatures.

Temperature	15 °C				20 °C			
Diets	D0	D0.5	D1	D2	D0	D0.5	D1	D2
Lysozyme (mg ml^{-1})	7.3 ± 3.4	6.8 ± 3.0	9.2 ± 2.1	7.6 ± 2.4	6.2 ± 2.4	4.8 ± 2.5	4.6 ± 1.8	4.3 ± 3.1
Ig (mg ml^{-1})	20.7 ± 5.4	20.0 ± 1.3	20.6 ± 3.1	20.3 ± 5.4	20.3 ± 4.6	18.2 ± 2.0	18.6 ± 4.6	21.3 ± 4.1
Peroxidase (U ml^{-1})	600.3 ± 173.5	444.3 ± 220.4	531.9 ± 373.1	437.0 ± 222.8	441.8 ± 113.2	510.6 ± 188.4	433.3 ± 214.4	346.2 ± 105.3
ACP	66.0 ± 5.2	66.5 ± 4.5	66.1 ± 6.3	66.1 ± 3.9	66.9 ± 4.1	64.1 ± 5.7	65.4 ± 5.3	66.0 ± 5.2

Two-way ANOVA

Variation source	Temperature	Diets	Interaction
Lysozyme (mg ml^{-1})	***	ns	ns
Ig (mg ml^{-1})	ns	ns	ns
Peroxidase (U ml^{-1})	ns	ns	ns
ACP	ns	ns	ns

Values presented as means ± standard deviation (±SD) ($n = 9$).
Two-way ANOVA: ***$P < 0.001$; ns: not significant.

Table 5
Specific activities of antioxidant enzymes and lipid peroxidation (LPO) levels of turbot fed the experimental diets at two temperatures.

Temperature	15 °C				20 °C			
Diets	D0	D0.5	D1	D2	D0	D0.5	D1	D2
SOD (U mg protein^{-1})	116.0 ± 16.0 b	106.4 ± 6.1 ab	102.6 ± 5.7 a	110.2 ± 7.5 ab	104.1 ± 9.2 a	104.8 ± 8.6 ab	115.1 ± 5.9 bc	123.8 ± 8.2 c
CAT (U mg protein^{-1})	75.6 ± 6.8 b	66.2 ± 4.6 a	61.2 ± 6.3 a	75.4 ± 2.7 b	60.5 ± 3.1 b	55.6 ± 2.8 a	55.8 ± 6.0 ab	51.9 ± 2.0 a
GPX (mU mg protein^{-1})	100.1 ± 19.5 ab	82.9 ± 24.5 a	110.1 ± 22.9 b	119.4 ± 12.2 b	69.1 ± 42.5 b	123.8 ± 12.3 c	95.8 ± 21.0 bc	20.8 ± 7.5 a
GR (mU mg protein^{-1})	5.5 ± 0.5 b	4.6 ± 0.3 a	4.8 ± 0.4 a	5.1 ± 0.5 ab	4.9 ± 0.2	4.9 ± 0.2	4.8 ± 0.4	4.7 ± 0.4
G6PDH (mU mg protein^{-1})	71.7 ± 5.1	67.6 ± 6.7	71.0 ± 13.4	62.4 ± 12.8	72.1 ± 5.2	64.2 ± 9.7	68.8 ± 8.6	70.5 ± 10.8
LPO (nmol MDA g tissue^{-1})	10.4 ± 1.0	11.1 ± 0.7	11.0 ± 0.9	10.9 ± 0.9	11.1 ± 0.6	10.9 ± 1.2	11.3 ± 0.8	11.3 ± 0.7

Two-way ANOVA

Variation source	Temperature	Diets	Interaction
SOD (U mg protein^{-1})	ns	**	***
CAT (U mg protein^{-1})	***	***	***
GPX (mU mg protein^{-1})	***	***	***
GR (mU mg protein^{-1})	ns	**	**
G6PDH (mU mg protein^{-1})	ns	ns	ns
LPO (nmol MDA g tissue^{-1})	ns	ns	ns

Values presented as means ± standard deviation (±SD) ($n = 9$).
Two-way ANOVA: $^{**}P < 0.01$; $^{***}P < 0.001$; ns: not significant. If interaction was significant, one-way ANOVA was performed for diets within each temperature and mean in the same line with different letters are significantly different ($P < 0.05$).

numbers decreased to control levels. Although lysozyme activity was still high after 50 days of feeding with MacroGard® the authors questioned the benefits of long-term use of prebiotics as immunostimulants [40]. Long-term immunostimulation may also shift away energy that otherwise would be used for normal growth and may thus result in lower animal productivity. That was apparently not the case in our study, as no differences in growth performances were observed after 9 weeks feeding the prebiotic diets [13]. Further studies assessing the time-course effect of scFOS supplementation on immune parameters are required to elucidate eventual immunostimulatory properties at short time.

In the present study, lysozyme activity was affected by rearing temperature, with higher levels being observed in fish reared at 15 °C, which is within the optimal temperature range for this species. A few other studies have also investigated the effects of temperature on teleost immunity. Innate immune parameters appear to present higher activity whereas adaptive parameters tend to be suppressed at lower temperatures [2]. However, contradictory results were observed both within and between species. For instance, decreased lysozyme levels during winter months were observed in Atlantic halibut (*Hippoglossus hippoglossus*) and European seabass (*Dicentrarchus labrax*) [41,42] whereas other studies did not observe changes in lysozyme activity in European seabass reared throughout the year [43]. Lysozyme is mainly produced by macrophages and neutrophils, and while WBC (%) was higher at 15 °C than at 20 °C, which is in line with the increased lysozyme activity at that temperature, neutrophils were lower at 15 °C, and no further differences in leucocytes count was observed related to temperature. Thus, it seems that the increased lysozyme values observed at 15 °C is related to increased activity in leucocytes rather than to increased leucocyte number.

To our knowledge, this is the first study evaluating the effect of prebiotic incorporation and rearing temperatures on oxidative stress. Studies accessing the antioxidant capabilities related to prebiotic use in fish are also scarce. In turbot, triangular bream and large yellow croaker (*Larimichthys crocea*) it was reported an increased antioxidant potential when fish were fed combinations of prebiotics and probiotics [8,10,11]. In the present study, fish presented different antioxidant enzymatic activities between the different feeding treatments and rearing temperatures. Results are however difficult to interpret as there was a significant interaction between temperature and scFOS for the enzymes activities. Still, the lack of differences in LPO levels, which is a biomarker of oxidative cellular damage [44–47] suggests that fish were able to efficiently counteract eventual differences in ROS produced at the two temperatures.

Although SOD is considered the first enzyme responding to the presence of oxygen radicals [48], in the present study SOD was not affected by the rearing temperature. Similar results were also observed by Ref. [15] in rohu (*Labeo rohita*) at 27 and 32 °C. However, both CAT and GPX were higher at 15 °C, which was somehow unexpected as higher ROS are expected to be formed at higher temperatures. Results also seem to indicate different optimal scFOS incorporation levels depending on the rearing temperature. Turbot reared at 15 °C presented lower activity of SOD, CAT and GR with 0.5 or 1% scFOS incorporation which seems to indicate a beneficial effect of scFOS on ROS production. On the other hand, fish reared at 20 °C presented lower CAT and GPX activities when fed 2% scFOS, though SOD activity increased with dietary scFOS.

In summary, results of the present study suggest that dietary scFOS had no effect on turbot juvenile's innate humoral parameters and hematology. High temperature (20 °C) does not seem to increase turbot oxidative stress comparatively to optimal rearing temperature (15 °C). Dietary scFOS seems to affect turbot's oxidative stress response, but effects are temperature related.

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Chapter 5
Effects of short-chain fructooligosaccharides (scFOS) and rearing temperature on growth performance and hepatic intermediary metabolism in gilthead sea bream (*Sparus aurata*) juveniles

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Effects of short-chain fructooligosaccharides (scFOS) and rearing temperature on growth performance and hepatic intermediary metabolism in gilthead sea bream (*Sparus aurata*) juveniles

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Abstract The effect of dietary short-chain fructooligosaccharides (scFOS) incorporation on growth, feed utilization, body composition, plasmatic metabolites and liver activity of key enzymes of lipogenic and amino acid catabolic pathways was evaluated in gilthead sea bream reared at 18 and 25 °C. Four practical diets containing plant ingredients and fish meal (50:50) as protein sources and supplemented with 0, 0.1, 0.25 and 0.5 % scFOS were fed to triplicate groups of fish for 8 weeks. Growth performance, feed efficiency and nitrogen retention were higher at 25 °C. In fish reared at 18 °C, there was a positive correlation between dietary scFOS concentration and growth. At 18 °C, liver glycogen was higher in fish fed the control diet, while at 25 °C it was higher in fish fed the 0.5 % scFOS diet. Plasma cholesterol LDL was lower in fish fed 0.25 % scFOS diet, and in fish reared at 18 °C plasma glucose was higher in fish fed the 0.1 % scFOS diet. Glucose 6-phosphate dehydrogenase, fatty acid synthetase and aspartate aminotransferase (ASAT) activities were higher in fish reared at 18 °C, whereas alanine aminotransferase activity was higher in fish reared at 25 °C. scFOS affected ASAT activity, which was lower in fish fed 0.25 % scFOS diet. Although, scFOS seemed to have no major effects on gilthead sea bream metabolism, the positive correlation between dietary prebiotic incorporation and growth at 18 °C indicates a beneficial effect of scFOS in fish reared at low temperatures.

Keywords Amino acid catabolism enzymes · Lipogenic enzymes · Plasmatic metabolites · Prebiotics · Temperature · Gilthead sea bream

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Introduction

Since the beginning of aquaculture production, one of the main priorities was to improve fish growth and feed efficiency, which might be obtained through improvements in fish nutrition and metabolism. Improvements in fish nutrition will lead to higher fish performance and health and will also contribute to the reduction in the aquaculture environmental impact. Functional non-nutritive compounds are increasingly being studied as a mean to improve fish performance, stress tolerance and disease resistance (Oliva-Teles 2012). Among these, prebiotics and probiotics are receiving considerable consideration as means to manipulate gastrointestinal (GI) microbiota, thus

improving GI morphology and microbiota balance (Merrifield et al. 2010; Oliva-Teles 2012; Merrifield and Carnevali 2014; Ringø et al. 2014). It was shown that GI microbiota can regulate the expression of 212 genes in the gut of zebrafish, some of them related to the promotion of nutrient metabolism (Rawls et al. 2004).

Prebiotics are defined as non-digestive feed ingredients that promote growth of beneficial GI microbiota and depress the proliferation of detrimental microbiota (Gibson and Roberfroid 1995). Among prebiotics, fructooligosaccharides (FOS) are one of the most tested in fish (Merrifield et al. 2010; Ringø et al. 2010, 2014; Dimitroglou et al. 2011; Hoseinifar et al. 2014; Zhang et al. 2014). However, studies conducted so far in fish have been focused mainly on FOS effects on growth performance, immune response and disease resistance (Grisdale-Helland et al. 2008; Buentello et al. 2010; Ai et al. 2011; Ye et al. 2011; Soleimani et al. 2012; Ortiz et al. 2013; Wu et al. 2013; Zhang et al. 2013, 2014; Hoseinifar et al. 2014; Guerreiro et al. 2014a, b, 2015b). To our knowledge, up to now only two studies aimed to access short-chain fructooligosaccharides (scFOS) effects on fish metabolism (Guerreiro et al. 2014a, 2015b). Comparatively to FOS, scFOS are characterized by having a shorter degree of polymerization; usually, for each glucose unit, there are 1–5 fructose units (Bornet et al. 2002). FOS, as other prebiotics, are not digested by fish but are fermented by GI microbiota, resulting in production of short-chain fatty acids (SCFAs). SCFAs are absorbed by the enterocytes, accounting for a large proportion of enterocytes energy needs, and stimulate the growth of GI beneficial bacteria including lactic acid bacteria (Mountfort et al. 2002; Merrifield et al. 2010). Therefore, FOS can indirectly modify fish metabolism through gut bacteria-fermented end products, mainly SCFAs.

Studies in mammals indicate that prebiotics affect lipid metabolism, reducing hepatic lipogenesis and concentration of liver and serum triglycerides and increasing HDL/LDL ratio (Delzenne et al. 2002, 2008; Teitelbaum 2009). Such a modulation of lipid metabolism was related to the colonic production of SCFAs, namely acetate and propionate generated through prebiotics fermentation by GI microbiota. However, the effects of prebiotics on lipid metabolism in fish were scarcely studied (Torrecillas et al. 2011, 2015; Guerreiro et al. 2014a, 2015b). Studies concerning the effect of prebiotics on amino acid metabolism are not frequent. Although scarce, one study performed in cats with a mixture of oligofructose and inulin reported a reduction in plasmatic aspartate aminotransferase (Verbrugghe et al. 2009). To the author's knowledge, only one study is available concerning prebiotic effects on amino acids metabolism in fish (Guerreiro et al. 2014a). Therefore, contrary to mammals, the effects of prebiotics on fish metabolism are scarcely studied and gaps still persist in various other aspects of prebiotic effects on fish, including the environmental influence on prebiotic effects as pointed out by Ringø et al. (2014).

Gut bacteria of homoeothermic animals thrive under fairly constant temperature conditions, but in fish, due to its heterothermic nature, gut bacteria are subjected to important temperature variations and this may affect autochthonous bacterial communities (Hagi et al. 2004; Denev et al. 2009). However, potential effects of temperature on prebiotic effects are also scarcely studied (Guerreiro et al. 2014a, b, 2015a).

Thus, the aim of the present study was to evaluate the effect of dietary supplementation with scFOS on growth performance and hepatic intermediary metabolism of gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures, one within the optimal rearing temperature range and the other below the optimum temperature.

Materials and methods

Diets composition

A basal diet was formulated to be isolipidic (18 % lipid) and isonitrogenous (46 % protein) using fish meal and plant ingredients (soybean meal, wheat gluten, corn gluten and wheat meal) as protein sources (at circa 50:50 of protein from fish meal: plant ingredients) and fish oil as the main lipid source. The basal diet served as the control diet (diet D0—control diet), and the experimental diets were produced by supplementation of the basal formulation with increasing levels of scFOS [at 0.1 % (diet D0.1), 0.25 % (diet D0.25) or 0.5 % (diet D0.5)]. scFOS are a commercially available prebiotic (PROFEED Maxflow; Jefo, France). The ingredients were thoroughly blended and extruded in a laboratory pellet mill

(California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2.0-mm die. Pellets were dried in an oven at 40 °C for 48 h and then stored in airtight bags until use. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Growth trial

Gilthead sea bream (*Sparus aurata*) juveniles obtained from a commercial fish farm (Maresa, S.A., Ayamonte, Huelva, Spain) were transferred to the Marine

Table 1 Ingredients and proximate composition of the experimental diets

	Diets			
	D0	D0.1	D0.25	D0.5
Ingredients (% dry weight)				
Fish meal[a]	31.4	31.4	31.4	31.4
Corn gluten[b]	5.0	5.0	5.0	5.0
Wheat gluten[c]	5.0	5.0	5.0	5.0
Wheat meal[d]	15.2	15.2	15.2	15.2
Soy meal[e]	25.0	25.0	25.0	25.0
scFOS[f]	–	0.1	0.25	0.5
Cellulose[g]	0.5	0.4	0.25	–
Cod liver oil	13.7	13.7	13.7	13.7
Bicalcium phosphate[h]	0.7	0.7	0.7	0.7
Vitamin mix[i]	1.0	1.0	1.0	1.0
Mineral mix[j]	1.0	1.0	1.0	1.0
Choline chloride (50 %)	0.5	0.5	0.5	0.5
Binder (Aquacube)[k]	1.0	1.0	1.0	1.0
Proximate analysis (% dry weight)				
Dry matter	87.4	87.3	89.1	88.5
Crude protein	45.8	46.6	45.7	46.6
Crude lipids	18.7	18.0	18.0	18.3
Ash	9.3	9.1	9.2	9.3
Starch	10.8	10.5	11.4	11.1
Gross energy (kJ g^{-1})[l]	20.1	19.9	19.9	20.1

DM dry matter, *CP* crude protein, *CL* crude lipid

[a] Steam dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 71.7 % DM; CL: 9.5 % DM)

[b] Sorgal, S.A. Ovar, Portugal (CP: 72.2 % DM; CL: 2.0 % DM)

[c] Sorgal, S.A. Ovar, Portugal (CP: 84.4 % DM; CL: 1.8 % DM)

[d] Sorgal, S.A. Ovar, Portugal (CP: 14.1 % DM; CL: 3.2 % DM)

[e] Sorgal, S.A. Ovar, Portugal (CP: 50.2 % DM; CL: 2.4 % DM)

[f] PROFEED Maxflow "Fructo-Oligosaccharides" (Jefo, France)

[g] Sigma-Aldrich, Sintra, Portugal

[h] Premix, Portugal (calcium: 24 %; total phosphorus: 18 %)

[i] Vitamins (mg kg^{-1} diet): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400

[j] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet)

[k] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate)

[l] Gross energy calculated based on theoretical values (CP: 23.6 kJ g^{-1}; CL: 39.5 kJ g^{-1}; carbohydrates: 17.2 kJ g^{-1})

Zoology Station, Porto University, Portugal. Fish were kept in quarantine for 1 month and then transferred to the experimental systems for adaptation to the experimental conditions during 15 days. Before the experimental period, fish were fed a commercial diet (48 % protein and 17 % lipids) and kept at 25.0 ± 1.0 °C. Thereafter, groups of 22 gilthead sea bream with an initial mean body weight of 32 ± 0.01 g were randomly distributed to two identical recirculating water systems each equipped with 12 cylindrical fibreglass tanks of 100 l water capacity and thermoregulated, using refrigerators and heaters, to 18.0 ± 0.5 and 25.0 ± 0.6 °C. Fish in juvenile stage were selected to this study since it is a stage that is characterized by a fast growth and fast answers to the provided stimuli. Tanks were supplied with continuous flow of filtered seawater (2.5–3.5 l min^{-1}) of 35 ± 1 g l^{-1} salinity, and dissolved oxygen was kept near saturation (7 mg l^{-1}). The experimental diets were randomly assigned to triplicate groups within each temperature. Fish were fed by hand, twice daily, 6 days a week, for 8 weeks, until apparent visual satiation. Utmost care was taken to avoid feed losses. The experiment was performed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Sampling

Five fish from the initial stock population were randomly sampled and stored at −20 °C for whole-body composition analysis. At the end of the trial, fish were unfed for 24 h, bulk-weighed, and three fish per tank were sampled for biological parameters (liver and visceral indices, $n = 9$) and whole-body composition analyses (fish pooled by tank, $n = 3$).

To minimize stress caused by manipulation, the remaining fish in each tank continued to be fed for 3 more days and then fish were randomly sampled 6 h after the morning meal for the remaining analysis (plasma metabolites, $n = 9$; liver composition analysis, $n = 9$; enzyme activity measurement, $n = 9$ from pools of 2 livers). Fish blood was collected from the caudal vein using heparinized tubes and then centrifuged, and the obtained plasma was frozen at −80 °C until analysis. Livers were collected for composition analysis and stored at −20 °C. For enzyme activity measurement, livers were excised and kept in pools of 2 livers, immediately frozen in liquid nitrogen and then stored at −80 °C until analysis.

Proximate analysis

Chemical analysis of the whole-fish carcasses and diets was performed following Association of Official Analytical Chemists methods (AOAC 2000). Dietary starch content was determined according to Beutler (1984). Hepatic glycogen and lipid contents were determined as described by Plummer (1987) and Folch et al. (1957), respectively.

Plasma metabolites

Plasma metabolites were determined using enzymatic colorimetric methods from Spinreact (Girona, Spain): cholesterol (kit cod. 1001090), LDL (kit cod. 41023), glucose (kit cod. 1001191), total lipids (kit cod. 1001270) and triglycerides (kit cod. 1001312). Plasma protein analysis was carried out using the bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, USA), kit cod. 23225.

Enzymes activity

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), fatty acid synthetase (FAS, EC 2.3.1.38), malic enzyme (ME, EC 1.1.1.40), glutamate dehydrogenase (GDH, EC 1.4.1.2), alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were assayed as described in Guerreiro et al. (2014a).

Enzyme activity was expressed as specific activity. One unit of enzyme activity was defined as the amount of enzyme that catalysis the hydrolysis of 1 μmol min^{-1} of substrate at assay temperature (37 °C). Protein concentration was determined by the Bradford method (Bradford 1976) using Sigma-Aldrich protein assay kit (ref. B6916) with bovine serum albumin as a standard.

Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was done by two-way ANOVA, using the SPSS 21 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were

tested for normality and homogeneity by the Shapiro–Wilk and Levene's test, respectively. When normality was not verified, data were transformed prior to ANOVA. In the case of interaction between factors, a one-way ANOVA analysis was performed for prebiotic level within each temperature. Significant differences among groups were determined by the Tukey's multiple range test. The probability level of 0.05 was used for rejection of the null hypothesis.

Results

All experimental diets were promptly accepted by the fish, and no pathological signs were observed during the trial. Mortality was very low (<2 %), and it was not affected by diet or rearing temperature (Table 2).

Fish reared at the higher temperature had higher final body weight, thermal growth unit (TGU), feed intake (FI), feed and protein efficiencies (FE and PER,

Table 2 Growth performance and feed utilization efficiency of gilthead sea bream fed the experimental diets at two temperatures

Temperature	18 °C				25 °C			
Diets	D0	D0.1	D0.25	D0.5	D0	D0.1	D0.25	D0.5
Final body weight (g)	62.8 ± 2.3	63.0 ± 2.9	65.2 ± 2.5	67.7 ± 1.9	90.1 ± 3.2	91.1 ± 5.2	92.8 ± 1.3	91.0 ± 2.4
Thermal growth unit[a]	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.10 ± 0.00	0.10 ± 0.00
Mortality (%)[b]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.52 ± 2.62	1.52 ± 2.62	1.52 ± 2.62	0.00 ± 0.00	0.00 ± 0.00
Feed intake (g kg ABW^{-1} day^{-1})[c]	17.7 ± 1.7	17.9 ± 1.0	19.3 ± 1.5	20.2 ± 2.4	24.0 ± 1.8	24.3 ± 1.5	23.9 ± 1.9	23.3 ± 1.1
Feed efficiency[d]	0.71 ± 0.05	0.70 ± 0.02	0.68 ± 0.02	0.70 ± 0.07	0.77 ± 0.01	0.76 ± 0.02	0.79 ± 0.06	0.79 ± 0.03
Protein efficiency ratio[e]	1.54 ± 0.10	1.50 ± 0.04	1.49 ± 0.42	1.49 ± 0.16	1.69 ± 0.03	1.63 ± 0.04	1.72 ± 0.14	1.70 ± 0.07
Nitrogen retention (% NI[f])	27.8 ± 2.4	26.4 ± 1.0	27.2 ± 0.9	26.9 ± 2.2	29.7 ± 1.9	28.8 ± 1.4	30.9 ± 2.4	30.8 ± 1.9

Two-way ANOVA

Variation source	Temperature	Diets	Interaction
Final body weight (g)	***	ns	ns
Thermal growth unit[a]	***	ns	ns
Mortality (%)[b]	ns	ns	ns
Feed intake (g kg ABW^{-1c} day^{-1})	***	ns	ns
Feed efficiency[d]	***	ns	ns
Protein efficiency ratio[e]	***	ns	ns
Nitrogen retention (% NI[f])	***	ns	ns

Values presented as mean ± standard deviation (±SD) ($n = 3$)

ns not significant

[a] Thermal growth unit (TGU): [(final body $weight^{1/3}$ − initial body $weight^{1/3}$)/(temperature × time in days)] × 100

[b] Mortality: (number of dead fish × 100/number of initial fish)

[c] ABW: average body weight (initial body weight + final body weight)/2

[d] Feed efficiency (FE): (wet weight gain/dry feed intake)

[e] Protein efficiency ratio (PER): (wet weight gain/crude protein intake)

[f] *NI* nitrogen intake

Two-way ANOVA: *** $P < 0.001$

respectively) and nitrogen retention (%NI, nitrogen intake) (NR) ($P < 0.05$) (Table 2). Dietary prebiotic supplementation had no effect on final weight, feed intake or feed efficiency ($P > 0.05$). Regarding fish performance, there was also no interaction between temperature and dietary prebiotic incorporation. However, a significant linear correlation between dietary prebiotic incorporation and fish final body weight (with $R = 0.98$ and $P = 0.01$) was found in fish reared at 18 °C.

Whole-body dry matter, lipid and ash content were higher in fish reared at 25 °C ($P < 0.05$), while protein was not affected by water temperature ($P > 0.05$) (Table 3). Visceral somatic indices (VSI) and hepatosomatic indices (HSI) were higher in fish reared at 18 °C, while liver lipid content was higher in fish reared at 25 °C ($P < 0.05$). Dietary prebiotic did not affect any of the above parameters ($P > 0.05$), but liver glycogen was affected by dietary prebiotic incorporation ($P < 0.05$). At 18 °C, fish fed the prebiotic diets had lower liver glycogen content, while at 25 °C liver glycogen content was higher in fish fed the 0.5 % scFOS diet (Table 3).

All plasmatic metabolites measured were affected by rearing temperature, but only cholesterol LDL, glucose, total lipids and protein levels were affected by dietary prebiotics (Table 4). Plasma cholesterol, glucose, total lipids and protein were higher in fish reared at 18 °C ($P < 0.05$), while cholesterol LDL and triglycerides were higher in fish reared at 25 °C ($P < 0.05$). Cholesterol LDL was lower in fish fed 0.25 % scFOS than in fish fed 0.5 % scFOS ($P < 0.05$). Plasma glucose was affected by dietary prebiotic only in fish reared at the lower temperature, being higher in fish fed diet D0.1 than the control diet ($P < 0.05$). On the contrary, plasma total lipids and proteins were only affected on fish reared at the higher temperature ($P < 0.05$). Thus, total lipids were higher in fish fed the 0.5 % scFOS diet, whereas total protein was higher in fish fed the control diet.

ME and GDH were not affected by rearing temperature or by dietary prebiotic ($P > 0.05$) (Table 5). G6PD, FAS and ASAT activities were higher in fish reared at 18 °C, and ALAT activity was higher in fish reared at 25 °C ($P < 0.05$). Dietary prebiotic only affected ASAT activity being lower in fish fed diet D0.25 than the control diet ($P < 0.05$) (Table 5).

Discussion

In the last years, several studies about prebiotics on fish have arisen, mainly reporting effects on growth performance, microbiota modifications, immune modulation and disease resistance (Merrifield et al. 2010; Ringø et al. 2010, 2014; Dimitroglou et al. 2011; Song et al. 2014; Torrecillas et al. 2014). However, topics such as prebiotic effects on fish metabolism or the influence of rearing temperature on prebiotics effect received poor attention (Guerreiro et al. 2014a, b, 2015a, b).

The effects of prebiotics on growth performance are dependent on aspects such as prebiotic source, fish species and diet composition, which may explain why some studies reported improved fish growth due to dietary FOS incorporation (Mahious et al. 2006; Zhou et al. 2010; Soleimani et al. 2012; Ortiz et al. 2013; Wu et al. 2013) while others reported no growth improvement (Grisdale-Helland et al. 2008; Buentello et al. 2010; Ye et al. 2011; Hoseinifar et al. 2014; Guerreiro et al. 2014a, 2015b), or even report adverse effects on fish performance (Hoseinifar et al. 2011a). In the present study, effect of dietary scFOS supplementation seems to be influenced by temperature. Indeed, in fish reared at 18 °C, but not in the ones reared at 25 °C, it was found a positive linear correlation between prebiotic incorporation level and fish final body weight. This suggests a beneficial effect of dietary scFOS on performance of fish reared under suboptimal temperatures.

In the present study, no FE improvement was observed due to dietary prebiotic supplementation. Similar results were also observed in studies with red drum (*Sciaenops ocellatus*) (Buentello et al. 2010; Zhou et al. 2010) fed FOS or turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*) fed scFOS (Guerreiro et al. 2014a, 2015b). However, enhanced FE due to diet prebiotic supplementation was observed in other studies (Li et al. 2008; Soleimani et al. 2012). This increased FE was attributed to an increase in exogenous microbial activity, which enhanced diet digestibility (Soleimani et al. 2012).

As in turbot (Guerreiro et al. 2014a), in the present study with gilthead sea bream temperature also did not influence scFOS effects on fish intermediary metabolism.

Table 3 Whole-body (wet weight basis, %), liver composition (g 100 g^{-1} liver), hepatosomatic and visceral somatic indices of gilthead sea bream fed the experimental diets at two temperatures

Temperature		18 °C				25 °C			
Diets	Initial	D0	D0.1	D0.25	D0.5	D0	D0.1	D0.25	D0.5
Whole body									
Dry matter	26.8	31.3 ± 0.9	29.9 ± 1.0	31.0 ± 0.2	31.2 ± 0.2	31.6 ± 0.9	32.5 ± 0.5	32.7 ± 0.7	32.4 ± 0.6
Protein	16.1	17.0 ± 0.2	16.8 ± 0.2	17.2 ± 0.1	17.1 ± 0.2	17.2 ± 0.5	17.1 ± 0.3	17.3 ± 0.2	17.4 ± 0.2
Lipid	7.4	11.3 ± 1.7	10.1 ± 1.6	11.0 ± 1.0	11.1 ± 0.2	11.5 ± 1.0	12.3 ± 0.5	12.2 ± 0.6	11.9 ± 0.7
Ash	5.65	3.85 ± 0.25	4.12 ± 0.10	4.12 ± 0.48	3.92 ± 0.08	4.43 ± 0.08	4.25 ± 0.34	4.25 ± 0.06	4.24 ± 0.21
VSI[a]	0.61	7.10 ± 0.94	6.71 ± 1.26	7.08 ± 1.18	7.47 ± 0.65	6.26 ± 0.65	5.86 ± 0.86	5.81 ± 0.80	6.15 ± 0.70
HSI[b]	4.51	1.99 ± 0.32	1.72 ± 0.29	2.02 ± 0.48	1.93 ± 0.42	1.19 ± 0.18	1.03 ± 0.15	1.20 ± 0.13	1.11 ± 0.14
Liver									
Lipid	–	4.90 ± 0.71	4.70 ± 0.54	5.02 ± 0.86	4.78 ± 1.07	6.19 ± 0.96	5.72 ± 1.13	5.90 ± 1.40	6.46 ± 1.25
Glycogen	–	12.3 ± 1.3 b	10.2 ± 1.3 a	10.7 ± 1.1 a	10.1 ± 1.1 a	10.1 ± 1.5 a	10.1 ± 1.0 a	11.5 ± 0.8 ab	11.5 ± 1.2 b

Two-way ANOVA

Variation source	Temperature	Diets	Interaction
Body			
Dry matter	***	ns	ns
Protein	ns	ns	ns
Lipid	*	ns	ns
Ash	*	ns	ns
VSI[a]	***	ns	ns
HSI[b]	***	ns	ns
Liver			
Lipid	***	ns	ns
Glycogen	ns	*	***

Values presented as mean ± standard deviation (±SD) ($n = 3$ for body composition, $n = 9$ for VSI, HSI and liver composition)

[a] Visceral somatic index: (viscera weight/body weight) × 100

[b] Hepatosomatic index: (liver weight/body weight) × 100

Two-way ANOVA: * $P < 0.05$; *** $P < 0.001$; *ns* not significant. If interaction was significant, one-way ANOVA was performed for prebiotics within each temperature and mean in the same line with different letters are significantly different ($P < 0.05$)

Table 4 Plasmatic cholesterol, cholesterol LDL, glucose (mg dL^{-1}), triglycerides, total lipids and total protein (g dL^{-1}) of gilthead sea bream fed the experimental diets at two temperatures

Temperature	18 °C				25 °C			
Diets	D0	D0.1	D0.25	D0.5	D0	D0.1	D0.25	D0.5
Cholesterol	0.23 ± 0.03	0.26 ± 0.05	0.25 ± 0.03	0.26 ± 0.03	0.23 ± 0.01	0.23 ± 0.02	0.23 ± 0.02	0.24 ± 0.02
Cholesterol LDL	0.13 ± 0.03	0.14 ± 0.02	0.12 ± 0.01	0.15 ± 0.02	0.14 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.16 ± 0.02
Glucose	64.4 ± 7.8 a	76.4 ± 6.0 b	72.2 ± 6.2 ab	72.5 ± 10.7 ab	66.4 ± 4.3	63.4 ± 6.1	66.1 ± 7.1	66.7 ± 6.8
Triglycerides	0.28 ± 0.09	0.33 ± 0.10	0.35 ± 0.09	0.31 ± 0.06	0.40 ± 0.12	0.39 ± 0.09	0.35 ± 0.08	0.52 ± 0.18
Total lipids	1.31 ± 0.16	1.37 ± 0.30	1.45 ± 0.16	1.42 ± 0.27	1.14 ± 0.16 a	1.17 ± 0.15 a	1.08 ± 0.10 a	1.42 ± 0.07 b
Total protein	3.6 ± 0.3	3.6 ± 0.3	3.6 ± 0.3	3.8 ± 0.3	3.7 ± 0.2 c	3.2 ± 0.2 a	3.6 ± 0.2 bc	3.3 ± 0.4 ab

Two-way ANOVA

Variation source	Temperature	Diets	Interaction	Diets			
				D0	D0.1	D0.25	D0.5
Cholesterol	**	ns	ns	–	–	–	–
Cholesterol LDL	*	*	ns	ab	ab	a	b
Glucose	**	ns	*				
Triglycerides	***	ns	ns	–	–	–	–
Total lipids	***	*	*				
Total protein	**	ns	**				

Values presented as mean ± standard deviation (±SD) ($n = 9$)

Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *ns* not significant. If interaction was significant, one-way ANOVA was performed for prebiotics within each temperature and mean in the same line with different letters are significantly different ($P < 0.05$)

Table 5 Specific activities (mU mg protein^{-1}) of lipogenic (glucose-6-phosphate dehydrogenase, G6PD, malic enzyme, ME and fatty acid synthetase, FAS) and amino acid catabolic enzymes (glutamate dehydrogenase, GDH, alanine aminotransferase, ALAT, aspartate aminotransferase, ASAT) in the liver of gilthead sea bream fed the experimental diets at two temperatures

Temperature	18 °C				25 °C			
Diets	D0	D0.1	D0.25	D0.5	D0	D0.1	D0.25	D0.5
G6PD	108.7 ± 13.0	109.5 ± 19.3	111.1 ± 13.0	100.2 ± 17.5	75.0 ± 12.9	73.2 ± 11.0	73.4 ± 10.5	79.5 ± 14.4
ME	2.8 ± 0.6	3.1 ± 0.5	2.9 ± 0.5	2.9 ± 0.7	2.6 ± 0.7	2.6 ± 0.6	3.1 ± 0.9	3.1 ± 0.6
FAS	7.3 ± 2.8	7.4 ± 3.8	8.2 ± 3.1	7.2 ± 3.6	3.6 ± 1.1	3.6 ± 1.2	4.2 ± 0.9	5.5 ± 2.1
GDH	65.9 ± 9.1	62.8 ± 5.8	66.2 ± 8.0	64.4 ± 8.2	62.3 ± 9.6	63.2 ± 11.8	61.8 ± 5.0	66.8 ± 9.9
ASAT	1443.5 ± 144.5	1358.9 ± 177.9	1321.3 ± 94.4	1355.2 ± 91.0	1336.3 ± 80.0	1322.4 ± 151.6	1218.0 ± 82.0	1309.1 ± 104.2
ALAT	475.8 ± 49.7	463.3 ± 64.7	450.0 ± 55.1	458.5 ± 52.6	517.6 ± 46.3	484.8 ± 39.2	490.1 ± 51.9	501.1 ± 57.2

Two-way ANOVA

Variation source	Temperature	Diets	Interaction	Diets			
				D0	D0.1	D0.25	D0.5
G6PD	***	ns	ns	–	–	–	–
ME	ns	ns	ns	–	–	–	–
FAS	***	ns	ns	–	–	–	–
GDH	ns	ns	ns	–	–	–	–
ASAT	*	*	ns	b	ab	a	ab
ALAT	**	ns	ns	–	–	–	–

Values presented as mean ± standard deviation (±SD) ($n = 9$)

Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *ns* not significant. Different letters stand for significant differences between diets

In streptozotocin-induced diabetic rats, FOS were able to improve glucose tolerance by reducing hyperglycaemia (Gobinath et al. 2010). Such an effect was not observed in the present study, whereas plasma glucose levels in prebiotic-fed fish were similar to the control group or even higher. In line with present data, no changes in glycaemia were observed in beluga (*Huso huso*) fed diets supplemented with oligofructose or inulin (Reza et al. 2009; Hoseinifar et al. 2011b).

Studies in mammals also reported that dietary FOS incorporation resulted in reduced hepatic lipogenesis, fat deposition and reduced plasmatic levels of triglycerides, cholesterol or total lipids (Gibson and Roberfroid 1995; Delzenne et al. 2002; Teitelbaum 2009). Accordingly, lower ME activity was recorded in turbot fed a diet supplemented with 2 % scFOS (Guerreiro et al. 2014a), and lower ME and G6PD activities were recorded in European sea bass fed diets with mannan oligosaccharides (Torrecillas et al. 2011). A reduction in plasma cholesterol level was observed in beluga fed a diet with 2 % oligofructose (Hoseinifar et al. 2011b). In contrast, present data on ME, FAS and G6PD activities pointed to an absence of dietary scFOS effect on lipogenesis. Also, contrary to what was reported in mammals, plasmatic total lipids were higher in fish fed 0.5 % scFOS at 25 °C when compared to fish fed control diet. It is speculated that alterations of lipogenic enzymes activities may be related to the fermentation of prebiotics by intestinal bacteria, which produces SCFAs such as propionate and acetate.

Propionate inhibits fatty acids synthesis while acetate is a lipogenic substrate (Delzenne et al. 2002; Teitelbaum 2009). Thus, an increase or a decrease in lipogenesis might be related to the relative proportions of these two end products of fermentation. Therefore, the lack of variation in lipogenetic enzymes activity reported in the present study might be related to the ratio of acetate/propionate producing bacteria that are present in fish GI tract and that deserves to be further investigated.

The effect of prebiotics on amino acids metabolism is scarce even in mammals. In cats fed a mixture of oligofructose and inulin, a reduction in plasmatic ASAT was observed, and the authors attributed it to a reduction in gluconeogenesis from amino acids (Verbrugghe et al. 2009). Guerreiro et al. (2014a) working with turbot observed lower hepatic GDH activity in fish fed 0.5 and 2 % scFOS, suggesting a potential protein sparing effect by scFOS. In the present study, dietary supplementation with 0.25 % scFOS lead to a significant reduction in hepatic ASAT activity, indicating that protein catabolism might have decreased with the inclusion of scFOS in the diet. However, total protein plasmatic levels were only significantly lower than the control diet in fish fed 0.1 and 0.5 % scFOS and reared at 25 °C. Moreover, GDH activity, a key enzyme in protein catabolism and N retention (% NI) were not affected, suggesting that protein catabolism was not influenced by the inclusion of scFOS in the diets.

Overall, results of the present study showed no major effects of scFOS dietary incorporation on gilthead sea bream metabolism. The positive correlation between dietary prebiotic incorporation and fish growth at 18 °C seems to indicate a beneficial effect of dietary scFOS incorporation when fish are reared at low temperatures, and this deserves to be further studied.

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Chapter 6
Effect of short chain fructooligosaccharides (scFOS) on immunological status and gut microbiota of gilthead sea bream (*Sparus aurata*) reared at two temperatures

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ABSTRACT

The effects of dietary short chain fructooligosaccharides (scFOS) incorporation on hematology, fish immune status, gut microbiota composition, digestive enzymes activities, and gut morphology, was evaluated in gilthead sea bream (*Sparus aurata*) juveniles reared at 18 °C and 25 °C. For that purpose, fish with 32 g were fed diets including 0, 0.1, 0.25 and 0.5% scFOS during 8 weeks. Overall, scFOS had only minor effects on gilthead sea bream immune status. Lymphocytes decreased in fish fed the 0.1% scFOS diet. Fish fed the 0.5% scFOS diet presented increased nitric oxide (NO) production, while total immunoglobulins (Ig) dropped in those fish, but only in the ones reared at 25 °C. Red blood cells, hemoglobin, bactericidal activity and NO were higher at 25 °C, whereas total white blood cells, circulating thrombocytes, monocytes and neutrophils were higher at 18 °C. In fish fed scFOS, lymphocytes were higher at 18 °C. Total Ig were also higher at 18 °C but only in fish fed 0.1% and 0.5% scFOS diets. No differences in gut bacterial profiles were detected by PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis) between dietary treatments. However, group's similarity was higher at 25 °C. Digestive enzymes activities were higher at 25 °C but were unaffected by prebiotics incorporation. Gut morphology was also unaffected by dietary prebiotic incorporation.

Overall, gut microbiota composition, digestive enzymes activities and immunity parameters were affected by rearing temperature whereas dietary scFOS incorporation had only minor effects on these parameters. In conclusion, at the tested levels scFOS does not seem worthy of including it in gilthead sea bream juveniles diets.

1. Introduction

Prebiotics can be defined as non-digestible fibers that potentially increase specific-health promoting gut bacteria in the host [1]. Thus, prebiotics can positively affect host's health either indirectly, through by-products produced during bacterial prebiotic fermentation, or directly, through prebiotics interaction with pattern recognition receptors [2].

Fructooligosaccharides (FOS) are one of the most studied prebiotics in humans, farm animals and fish [2–5]. Short-chain fructooligosaccharides (scFOS) are similar to FOS but with a lower degree of polymerization, ranging from 1 to 5 fructose oligomers [6]. ScFOS are however much less studied than FOS. In fish, scFOS has been only evaluated in turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), common carp (*Cyprinus carpio*), and hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) [7–14].

FOS is known to support growth and survival of gastrointestinal tract autochthonous bacteria, such as members of the genus *Lactobacillus*, which possess β-fructosidase activity and thus can hydrolyse FOS β-(2-1) glycosidic bonds [3]. *Lactobacillus* is known to interact with the host immune system, but the precise mechanisms involved are not completely clarified. Nonetheless, it seems that *Lactobacillus*, or its end-metabolic products, interact with gut

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epithelial cells, macrophages, dendritic cells, and lymphocytes [15]. For instance, in mice given *Lactobacillus* as a probiotic, the gut mucosal immune system was affected mainly through activation of innate immune response cells [16]. FOS is also considered an immunosaccharide [2] as it has a direct signaling capacity on human's immune cells, by activating toll-like receptors, mainly TLR2 and, to a lesser extent, TLR4 [17]. Although the benefits of prebiotics to animals are well known, as well as the relationship between gut bacteria and host's immune system, the majority of studies on prebiotics effects in fish immune status does not simultaneously evaluate gut microbial composition [18–23]. It was, however, recently reported an increase in gut cultivable lactic acid bacteria (LAB) population and a stimulation of several immune parameters upon incorporation of FOS in the diets for stellate sturgeon (*Acipenser stellatus*) and common carp (*C. carpio*) [24,25].

Prebiotics effects on fish immunity, particularly of FOS, are reported as immunomodulatory [2–5]. For instance, blunt snout bream (*Megalobrama amblycephala*) fed a diet with 0.4% FOS presented lower levels of plasma cortisol and higher levels of immunoglobulin, lysozyme, plasma acid phosphatase, alternative complement activity and nitrogen monoxide than fish fed the control diet [23]. Triangular bream (*Megalobrama terminalis*) fed a diet with 0.6% FOS had increased leucocyte counts, plasma alternative complement activity and immunoglobulins compared to fish fed the control diet [22]. Red drum (*Sciaenops ocellatus*) fed a diet with 1% FOS had increased plasma lysozyme [26]. In Pacific white shrimp (*Litopenaeus vannamei*), diets including scFOS led to alterations in gut microbiota and enhanced total hemocyte count and hemocyte respiratory burst [27].

In gilthead sea bream, prebiotics effects on immune parameters were so far only evaluated for inulin and mannanoligosaccharides (MOS). MOS did not affect fish health indicators, whereas leucocytes phagocytic capacity was decreased in fish fed inulin for 1 week [18,21]. In another study, complement activity, leukocyte phagocytic ability and capacity increased after 2 weeks of feeding an inulin supplemented diet [28]. However, after 4 weeks of feeding the same diet, differences in leucocytes phagocytic ability and capacity disappeared while gut bacteria richness was reduced [28,29].

Dietary supplementation with FOS has been associated with an increase in digestive enzymes activity in some fish species, which may be correlated with alterations in gut microbiota [30–32]. Although, the effect of FOS on gilthead sea bream digestive enzymes was not yet evaluated, MOS was associated with increased protein, carbohydrates and energy digestibility's [33].

Prebiotic effects on fish gut morphology are extensively studied. MOS was reported to increase gut absorptive area through increased microvilli length and density [34–38]. FOS was also reported to induce changes in morphology of fish intestine, such as increased microvillus height [26,32]. However, inulin was reported to induce significant damage in gilthead sea bream gut [29].

Fish, as heterothermic animals, are heavily influenced by environmental conditions and, as suggested by Ringø et al. [3], temperature may have greater effects than diet in fish health. This may turn difficult an evaluation of prebiotics effect. Indeed, water temperature was already reported to influence immunological parameters and gut bacterial community [9,12,39–42].

Several studies on the effects of prebiotics [2,9,43] and rearing temperature [9,41,42] on fish immune status are already available. However, studies simultaneously evaluating both parameters and their potential interactive effects on fish immune status are very scarce [9].

Since prebiotics are reported to promote gastrointestinal health and immunological status [44], the study of prebiotic effects on gut function and integrity, and on immunological parameters are particularly important. Therefore, the aim of this study was to evaluate the effect of dietary scFOS supplementation in the hematological profile, fish immune status, allochthonous gut microbiota composition, digestive enzymes activities, and gut morphology of gilthead sea bream juveniles reared at two temperatures: 18 and 25 °C.

2. Material and methods

2.1. Diets composition

Four diets were formulated to be isolipidic (18% lipid) and isonitrogenous (46% protein). Fish meal and plant feedstuffs (soybean meal, wheat gluten, corn gluten and wheat meal) were used as protein sources (circa 50% protein from fish meal and 50% from plant feedstuffs), and fish oil was the main lipid source. The experimental diets included 0% (diet D0 – control diet), 0.1% (diet D0.1), 0.25% (diet D0.25), and 0.5% (diet D0.5) of scFOS (PROFEED Maxflow, Jefo, France) replacing α-cellulose. All ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2.0 mm die. Pellets were dried in an oven at 40 °C for 48 h, and then stored in a freezer in airtight bags until use. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Chemical analyses of the diets were performed following the Association of Official Analytical Chemists methods [45]. Dietary starch content was determined according to Beutler [46].

2.2. Growth trial

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with gilthead sea bream (*S. aurata*) juveniles obtained from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). The trial was performed in 2 identical recirculating water systems, each equipped with 12 cylindrical fiberglass tanks of 100 L water capacity, and thermo-regulated to 18.0 ± 0.5 °C and 25.0 ± 0.6 °C, respectively. The tanks were supplied with a continuous flow of filtered seawater (2.5–3.5 L min^{-1}) of 35 ± 1 g L^{-1} salinity, and dissolved oxygen was kept near saturation (7 mg L^{-1}). After a quarantine period of 1 month, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. During quarantine and adaptation periods, fish were fed a commercial diet (48% protein and 17% lipids; Sorgal, S.A. Ovar, Portugal). A total of 528 fish with an initial mean body weight of 32.0 ± 0.01 g were randomly distributed by the tanks, 22 fish per tank. The experimental diets were randomly assigned to triplicate groups within each temperature, hence 6 tanks per dietary treatment, with 3 tanks at each temperature. The trial lasted 8 weeks, and during that period fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. The experiment was performed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

2.3. Sampling

At the end of the trial, a total of 8 fish per tank were randomly sampled 4 h after the morning meal. Blood from 3 fish was collected from the caudal vein using heparinized syringes and placed in heparinized tubes. One aliquot was used for hematological assessment while the remaining blood was centrifuged at 3000 × g for 10 min at room temperature. The resulting plasma was

Table 1
Ingredients and proximate composition of the experimental diets.

	Diets			
	D0	D0.1	D0.25	D0.5
Ingredients (% dry weight)				
Fish meal[a]	31.4	31.4	31.4	31.4
Corn gluten[b]	5.0	5.0	5.0	5.0
Wheat gluten[c]	5.0	5.0	5.0	5.0
Wheat meal[d]	15.2	15.2	15.2	15.2
Soy meal[e]	25.0	25.0	25.0	25.0
scFOS[f]	–	0.1	0.25	0.5
Cellulose[g]	0.5	0.4	0.25	–
Cod liver oil	13.7	13.7	13.7	13.7
Bicalcium phosphate[h]	0.7	0.7	0.7	0.7
Vitamin mix[i]	1.0	1.0	1.0	1.0
Mineral mix[j]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder (Aquacube)[k]	1.0	1.0	1.0	1.0
Proximate analysis (% dry weight)				
Dry matter	87.4	87.3	89.1	88.5
Crude protein	45.8	46.6	45.7	46.6
Crude lipids	18.7	18.0	18.0	18.3
Ash	9.3	9.1	9.2	9.3
Starch	10.8	10.5	11.4	11.1
Gross energy (kJ g^{-1})[l]	20.1	19.9	19.9	20.1

DM, dry matter; CP, crude protein; CL, crude lipid.
[a] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 71.7% DM; CL: 9.5% DM).
[b] Sorgal, S.A. Ovar, Portugal (CP: 72.2% DM; CL: 2.0% DM).
[c] Sorgal, S.A. Ovar, Portugal (CP: 84.4% DM; CL: 1.8% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 14.1% DM; CL: 3.2% DM).
[e] Sorgal, S.A. Ovar, Portugal (CP: 50.2% DM; CL: 2.4% DM).
[f] PROFEED Maxflow "Fructo-Oligosaccharides" (Jefo, France).
[g] Sigma-Aldrich, Sintra, Portugal.
[h] Premix, Portugal (Calcium: 24%; Total phosphorus: 18%).
[i] Vitamins (mg kg^{-1} diet): retinol acetate, 18000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[j] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).
[k] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).
[l] Gross energy calculated based on theoretical values (CP: 23.6 kJ g^{-1}; CL: 39.5 kJ g^{-1}; Carbohydrates: 17.2 kJ g^{-1}.

frozen at −80 °C for immune parameters analyses. After blood collection fish were euthanized with a sharp blow in the head. Gut with intestinal content was removed and divided in 2 parts, anterior and posterior gut, immediately frozen in liquid nitrogen and then stored at −80 °C until measurement of digestive enzymes activities. The anterior gut was defined as the region from the last pyloric caeca and gut mid length. The posterior gut was defined as the second half of the gut.

Three other fish were euthanized with a sharp blow in the head, and gut was dissected on chilled trays and freed from the adjacent adipose and connective tissues. Circa 1 cm of the middle regions of the anterior and posterior gut were collected for histological evaluation. The samples were rinsed in phosphate buffered saline (PBS), carefully blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%, pH 7.4) for 24 h and then transferred to ethanol (70%) until further processing.

Two other fish per tank were sampled under aseptic conditions for digesta collection. Digesta was collected by squeezing the entire gut, immediately frozen in liquid nitrogen and then stored at −80 °C until characterization of allochthonous microbiota.

2.4. Hematological analysis

Fresh heparinized blood was used for hematocrit (Ht) and hemoglobin (Hb) determination, and for blood cells counts. Ht, Hb, total red blood cells (RBC), white blood cells (WBC) and differential white blood cell counts were determined as described by Guerreiro et al. [9]. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows:

$$MCV\ (\mu m^3) = (Ht/RBC) \times 10$$
$$MCH\ (pg\ cell^{-1}) = (Hb/RBC) \times 10$$
$$MCHC\ (g\ 100\ ml^{-1}) = (Hb/Ht) \times 100$$

2.5. Immune parameters

Bactericidal and anti-protease activities, nitric oxide (NO), and total immunoglobulins (Ig) in plasma were determined as described by Guerreiro et al. [9] and Machado et al. [47].

2.6. Microbial diversity

Samples of 2 fish per tank were pooled to reduce variation. DNA was extracted from 300 mg of gut contents as described in Pitcher et al. [48]. Bacterial 16S rRNA gene fragments were amplified by a touchdown PCR on a T100™ Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal), using oligonucleotide primers 16S-358F (which contained a GC clamp at the 5énd) and 16S-517R [49]. 300 ng of each PCR product were resolved on 8% polyacrylamide gel composed by a denaturing gradient of 40–80% 7 M urea/40% formamide. Electrophoresis was run on a DCode™ universal mutation detection system (Bio-Rad Laboratories Lda., Amadora, Portugal) during 16 h at 60 °C, 65 V in 1 × TAE buffer. Gels were stained for 1 h with SYBR-Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific Inc.) and imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal). Distinct bands were excised from the gel and eluted in 20 μl ultrapure water prior to DNA re-amplification [49] using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov) (Macrogen Europe, Amsterdam, The Netherlands). Only sequences higher than 100 bp reads and 80–100% query coverage were considered a valid identification.

2.7. Digestive enzymes activities

Each gut section was homogenized in 10 parts of ice-cold 50 mM Tris–HCl buffer pH 7.5. Homogenates were centrifuged at 33 000 × g for 15 min at 4 °C and the resultant supernatants were used for digestive enzyme assays.

Total alkaline protease (TAP), lipase (EC3.1.1.3) and α-amylase (EC3.2.1.1) activities, and protein concentrations were measured as described in Guerreiro et al. [12].

All enzyme activities were expressed as specific activity (U mg^{-1} of soluble protein for TAP and mU mg^{-1} for lipase and α-amylase).

2.8. Histological processing and morphological evaluation

Gut samples were processed and evaluated as described in Couto et al. [50] by visualization with a Carl Zeiss MicroImaging GmbH model Primo Star (Jena, Germany). Briefly, blinded

evaluation was performed using a semi-quantitative scoring system ranging from 1 to 5 (Table 2). Score 1 was given to the normal tissue appearance and subsequent scores accounted for increasing alterations in tissue histomorphology. Gut samples were evaluated according to the criteria suggested by Krogdahl et al. [51]: widening and shortening of the gut folds, loss of supranuclear vacuolization in absorptive cells (enterocytes) of the gut epithelium, nucleus position in the enterocyte, widening of lamina propria within gut folds, infiltration of mixed leucocyte population in lamina propria and submucosa. The overall score of histomorphological alterations was calculated by averaging scores of the separate parameters analyzed.

2.9. Statistical analysis

Data are presented as means ± standard deviation. Statistical analysis of hematological and immune parameters, and of digestive enzymes was done by two-way ANOVA, with diet and temperature as factors, using a SPSS 21 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were tested for normality by the Shapiro–Wilk test and for homogeneity of variances by the Levene's test. When normality was not verified data were transformed prior to ANOVA. In case of interaction between factors (Ht, lymphocytes, Ig, and posterior gut TAP), one-way ANOVA was performed for prebiotic level within each temperature. Significant differences among dietary groups were determined by the Tukey's multiple range test. In the case of interaction, t-tests were performed to assess differences between temperatures within the same diet. Histological data was neither normal nor homogeneous and could not be normalized, thus Kruskal–Wallis non-parametric tests and subsequent pairwise comparison were performed. The probability level of 0.05 was used for rejection of the null hypothesis in all tests.

DGGE (Denaturing Gradient Gel Electrophoresis) banding patterns were transformed into presence/absence matrices and band intensities were measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories Lda., Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef's measure of richness, and species diversity was assessed by the Shannon–Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the unweighted pair group method with arithmetic averages (UPGMA). Microbial diversity parameters were subjected to two-way ANOVA, with temperature and diet as fixed factors.

3. Results

3.1. Hematological parameters

Dietary prebiotic incorporation did not affect total blood cell counts, Ht, Hb, MCV, MCH, and MCHC (Table 3). MCV, MCH, and MCHC were also unaffected by rearing temperature. In contrast, RBC, and Hb were higher in fish reared at 25 °C whereas the opposite was observed for WBC. Ht was also higher in fish reared at 25 °C for all diets (t-test $P < 0.05$), except diet D0.1 (t-test $P > 0.05$).

Differential WBC counts are presented in Table 4. Thrombocytes, monocytes and neutrophils were higher in fish reared at 18 °C than at 25 °C. In fish fed scFOS diets (t-test $P < 0.05$), but not the control diet (t-test $P > 0.05$), lymphocytes were higher at 18 °C than at

Table 2
Continuous scale scoring system with the range of tissue scores set at 1–5, 5 indicating major alterations [52].

	Score range	
	1	to 5
Gut folds	Tall and distinct	Short, indistinct, fused
Lamina propria width and cellularity	Thin, low cellularity	Markedly widened and increased cellularity
Submucosa width and cellularity	Thin, low cellularity	Markedly widened and increased cellularity
Intraepithelial leucocytes infiltration	Low infiltration	Highly infiltrated
Enterocytes vacuolization	Well defined, regular vacuoles size	Absent or hypervacuolated; irregular vacuoles size
Enterocytes nucleus position	Basal	Apical

Table 3
Red (RBC) and white (WBC) blood cells counts, hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets				Two-way ANOVA[a]		
		D0	D0.1	D0.25	D0.5	Temperature	Diets	Interaction
RBC ($\times 10^6$ mm^{-3})	18 °C	2.17 ± 0.27	2.88 ± 1.21	2.21 ± 0.38	2.43 ± 0.22	*	ns	ns
	25 °C	2.68 ± 0.28	2.65 ± 0.31	2.50 ± 0.53	2.76 ± 0.62			
Ht (%)	18 °C	23.7 ± 2.1	26.3 ± 3.2	25.4 ± 2.3	26.6 ± 1.8	***	ns	*
	25 °C	29.1 ± 2.0	27.2 ± 3.1	30.4 ± 1.4	29.6 ± 2.9			
Hb (g dl^{-1})	18 °C	6.06 ± 0.47	6.36 ± 0.67	6.43 ± 0.59	6.50 ± 0.44	***	ns	ns
	25 °C	7.19 ± 0.65	7.00 ± 0.91	7.07 ± 1.12	7.23 ± 1.44			
MCV (μm^3)	18 °C	110.1 ± 13.1	99.5 ± 31.7	115.4 ± 16.5	110.1 ± 11.1	ns	ns	ns
	25 °C	109.4 ± 10.5	103.8 ± 13.2	129.7 ± 42.7	109.5 ± 16.1			
MCH (pg cell^{-1})	18 °C	28.2 ± 3.0	24.0 ± 7.4	29.3 ± 5.0	26.9 ± 2.9	ns	ns	ns
	25 °C	27.0 ± 2.6	26.6 ± 3.3	28.8 ± 3.5	26.4 ± 3.9			
MCHC (g 100 mL^{-1})	18 °C	25.7 ± 1.1	24.3 ± 0.8	25.4 ± 1.6	24.5 ± 1.4	ns	ns	ns
	25 °C	24.7 ± 1.3	25.7 ± 2.0	23.2 ± 3.4	24.3 ± 3.5			
WBC ($\times 10^4$ mm^{-3})	18 °C	14.7 ± 5.0	15.4 ± 3.4	15.8 ± 2.8	14.9 ± 2.3	***	ns	ns
	25 °C	6.01 ± 0.63	5.92 ± 0.58	6.46 ± 1.48	6.02 ± 1.16			

Values presented as means ± standard deviation (n = 9).
*P < 0.05; ***P < 0.001; ns: not significant.
[a] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for diets within each temperature.

Table 4
Differential white blood cells counts (thrombocytes, lymphocytes, monocytes and neutrophils ($\times 10^4$ mm^{-3})) of gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets				Two-way ANOVA[a]		
		D0	D0.1	D0.25	D0.5	Temperature	Diets	Interaction
Thrombocytes	18 °C	11.1 ± 4.3	11.7 ± 3.0	12.2 ± 3.0	11.5 ± 1.8	***	ns	ns
	25 °C	3.43 ± 1.11	4.30 ± 0.82	4.50 ± 1.46	4.13 ± 0.60			
Lymphocytes	18 °C	1.77 ± 0.78	2.37 ± 0.89	2.11 ± 0.96	2.22 ± 0.76	***	ns	**
	25 °C	1.89 ± 0.66 b	0.96 ± 0.54 a	1.19 ± 0.51 ab	1.26 ± 0.81 ab			
Monocytes	18 °C	0.81 ± 0.89	0.47 ± 0.29	0.62 ± 0.41	0.44 ± 0.20	***	ns	ns
	25 °C	0.23 ± 0.07	0.21 ± 0.11	0.21 ± 0.13	0.16 ± 0.10			
Neutrophils	18 °C	1.00 ± 0.73	0.91 ± 0.40	0.84 ± 0.48	0.72 ± 0.53	***	ns	ns
	25 °C	0.45 ± 0.09	0.44 ± 0.47	0.56 ± 0.22	0.47 ± 0.32			

Values presented as means ± standard deviation (n = 9).'
**P < 0.01; ***P < 0.001; ns: not significant.
[a] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for diets within each temperature, and values in the same line with different letters are significantly different (P < 0.05).

25 °C. Further, at 25 °C, lymphocytes decreased in fish fed diet D0.1 compared to fish fed the control diet.

3.2. Immune parameters

Plasma anti-protease activity was unaffected by rearing temperature or prebiotic supplementation, while plasma bactericidal activity and NO were higher in fish reared at 25 °C (Table 5). Plasma total Ig was also affected by rearing temperature, however due to interaction between factors, temperature affected diets differently. Thus, Ig was higher at 18 °C than at 25 °C in fish fed diets D0.1 and D0.5 (t-test P < 0.05), whereas temperature had no effect in fish fed the control and D0.25 diets (t-test P > 0.05). NO production was higher in fish fed diet D0.5 than the control and D0.25 diets. At 18 °C plasma total Ig was not affect by diet composition, while at 25 °C Ig was lower in fish fed diet D0.5 than the control and D0.25 diets.

3.3. Microbial diversity

The Bray–Curtis dendrogram and V3 16S rRNA DGGE fingerprints of bacterial communities indicate that the allochthonous (Fig. 1) population had more similar profiles at 25 °C (similarity percentages > 60%) than at 18 °C (similarity percentages ≈50%). Moreover, at 25 °C dietary treatments appeared to be clustered, while at 18 °C dietary treatments clusters were not observed.

The average number of OTUs, microbial richness, diversity, and similarity were not affected by dietary prebiotic (Table 6). Similarity was the only parameter affected by rearing temperature, with higher similarity registered in fish reared at 25 °C.

Sequence analysis from the DGGE bands (Table 7) showed that the detected dominant allochthonous bacteria were closely related to uncultured bacteria previously isolated from weaned piglets, wild and domesticated adult black tiger shrimp (*Penaeus monodon*), hybrid tilapia (*Tilapia nilotica* × *T. aurea*), and Canada goose feces. Three OTUs were most closely related to *Lactobacillus* sp., *Oceanobacillus* sp. and *Cyanothece* sp., while thirteen OTUs were not successfully sequenced.

3.4. Digestive enzymes

TAP, α-amylase and lipase activities in anterior and posterior gut are presented in Table 8 α-amylase and lipase activities were higher in fish reared at 25 °C, in both anterior and posterior gut. TAP activity was not affected by temperature in the anterior gut, but it was higher at 25 °C (t-test P < 0.05) in the posterior gut, except for fish fed diet D0.1 (t-test P > 0.05). TAP in the posterior gut was the only digestive enzyme affected by prebiotic incorporation. Thus, at 18 °C TAP activity was higher in fish fed diet D0.1 compared to fish fed diet D0.5.

3.5. Gut morphology

The average scores of parameters used to assess gut morphology were unaffected by prebiotic incorporation or rearing temperature (Table 9).

4. Discussion

It is known that interactions between gut bacteria and host

Table 5
Plasma immune humoral parameters of gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets				Two-way ANOVA[a]		
		D0	D0.1	D0.25	D0.5	Temperature	Diets	Interaction
Bactericidal Activity (%)	18 °C	45.7 ± 8.0	46.6 ± 2.9	44.6 ± 6.0	42.5 ± 5.3	***	ns	ns
	25 °C	59.6 ± 8.9	57.2 ± 7.3	63.9 ± 8.2	59.4 ± 8.1			
Nitric oxide (μM)	18 °C	88.4 ± 22.6 a	106.4 ± 20.8 ab	90.7 ± 26.1 a	105.9 ± 35.8 b	***	*	ns
	25 °C	113.9 ± 13.4 a	117.3 ± 19.7 ab	106.4 ± 17.1 a	139.1 ± 23.5 b			
Anti-protease Activity (%)	18 °C	76.5 ± 8.5	76.4 ± 7.1	76.6 ± 6.7	77.5 ± 5.1	ns	ns	ns
	25 °C	79.4 ± 8.2	77.6 ± 7.1	77.6 ± 9.5	77.1 ± 5.0			
Total Immunoglobulin (mg ml^{-1})	18 °C	18.8 ± 1.6	20.0 ± 3.1	19.3 ± 2.8	20.1 ± 2.2	***	ns	*
	25 °C	18.3 ± 1.5 b	17.2 ± 1.8 ab	18.0 ± 3.0 b	15.2 ± 2.0 a			

Values presented as means ± standard deviation (n = 9).
*P < 0.05; ***P < 0.001; ns: not significant.
[a] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for diets within each temperature, and values in the same line with different letters are significantly different (P < 0.05).

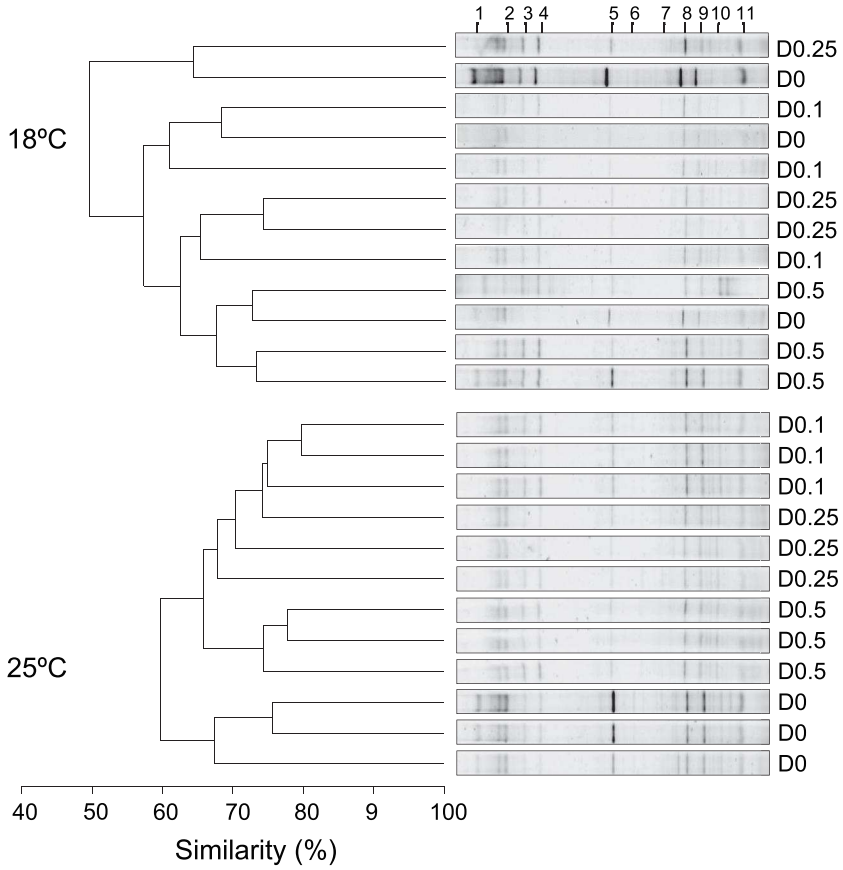

Fig. 1. Dendrogram and PCR-DGGE fingerprints of the allochthonous gut microbiota of gilthead sea bream fed the experimental diets at two temperatures. Numbers on top of the figure (1–11) indicate bands excised for sequence analysis.

Table 6
Ecological parameters obtained from PCR-DGGE fingerprints of gut allochthonous microbiota of gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets				Two-way ANOVA		
		D0	D0.1	D0.25	D0.5	Temperature	Diets	Interaction
OTUs[a]	18 °C	27.3 ± 4.5	23.3 ± 4.0	26.7 ± 6.0	28.0 ± 2.0	ns	ns	ns
	25 °C	32.0 ± 3.5	28.0 ± 2.0	25.3 ± 2.5	25.3 ± 3.1			
Richness[b]	18 °C	1.55 ± 0.22	1.35 ± 0.23	1.53 ± 0.32	1.60 ± 0.11	ns	ns	ns
	25 °C	1.82 ± 0.20	1.61 ± 0.12	1.46 ± 0.14	1.46 ± 0.18			
Diversity[c]	18 °C	3.27 ± 0.14	3.11 ± 0.16	3.23 ± 0.21	3.29 ± 0.06	ns	ns	ns
	25 °C	3.43 ± 0.10	3.30 ± 0.07	3.21 ± 0.09	3.20 ± 0.12			
SIMPER Similarity (%)[d]	18 °C	53.2 ± 12.9	57.7 ± 4.1	55.0 ± 16.7	70.2 ± 2.6	**	ns	ns
	25 °C	70.1 ± 5.0	76.5 ± 2.9	69.1 ± 1.8	75.5 ± 3.0			

Values presented as means ± standard deviation (n = 3 per treatment pooled from 6 fish).
**P < 0.01; ns: not significant.
[a] OTUs: average number of operational taxonomic units.
[b] Margalef species richness: d = (S−1)/log(N).
[c] Shannons diversity index: H′ = −∑(pi(lnpi)).
[d] SIMPER: similarity percentage within group replicates.

immune system affect host health [15,16]. Still, most studies on prebiotics effects in fish performance and immune status did not evaluate gut microbiota [18–23]. Results of the present study failed to show any interaction between gut microbiota composition and fish immune status in relation to dietary prebiotics.

The hematological profile was also almost unaffected by dietary scFOS. In fish, studies on prebiotic effects on differential WBC counts reported either lack of effects [24,25] or increased lymphocytes number [53]. On the contrary, in the present study, fish fed the 0.1% scFOS diet had a lower number of circulating lymphocytes, but this effect was only observed at 25 °C. In fish fed the scFOS, but not the control diet, temperature also affected lymphocytes numbers, which were higher in fish reared at 18 °C. This evidences the importance of rearing temperature in prebiotics action mode and deserves to be further investigated.

Prebiotics fermentation by gut bacteria produces short chain fatty acids (SCFAs) such as propionic, acetic, and butyric acid. Moreover, different gut bacteria lead to the production of different proportions of SCFAs [54]. SCFAs such as butyric acid are known to have immunomodulatory effects, and butyric acid was already

Table 7
Identified bacterial species from the DNA sequencing of the allochthonous gut communities of gilthead sea bream fed the experimental diets at two temperatures.

Band	Nearest neighbor	Similarity to nearest neighbor	Accession number of nearest neighbor
1	Uncultured bacteria from fermented foods such as pearl millet slurries	84	FN775030.1
2	Uncultured bacterium isolate DGGE gel band from the jejunum, ileum and cecum of weaned piglets	95	JX183818.1
3	Uncultured bacterium clone from Antarctic soil	86	HM710432.1
4	*Lactobacillus aviarius*	97	M58808.2
5	Uncultured bacterium clone from wild and domesticated adult black tiger shrimp (*Penaeus monodon*)	93	KF337424.1
6	Uncultured bacterium clone from hybrid tilapia (*Tilapia nilotica* × *T. aurea*)	90	EF599660.1
7	Uncultured *Cyanothece* sp. clone from rhizosphere of *Arachi hypogaea*	87	JX628832.1
8	*Oceanobacillus* sp.	94	KF958190.1
9	Uncultured bacterium clone from Canada goose feces	97	FJ390662.1
10	Uncultured bacterium isolate DGGE gel band from the jejunum, ileum and cecum of weaned piglets	99	JX183818.1
11	Uncultured bacterium clone from subsurface saline soil in Sanhu region of Qaidam Basin on Tibetan Plateau, China	90	GU212441.1

Table 8
Specific activities of digestive enzymes, total alkaline protease (U mg protein^{-1}), α-amylase and lipase (mU mg protein^{-1}) of anterior and posterior gut from gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets				Two-way ANOVA[a]		
		D0	D0.1	D0.25	D0.5	Temperature	Diets	Interaction
Anterior gut								
Total Alkaline Protease	18 °C	353.0 ± 75.8	284.4 ± 108.2	274.4 ± 79.5	364.3 ± 89.2	ns	ns	ns
	25 °C	371.4 ± 112.1	332.0 ± 125.5	303.0 ± 163.3	269.3 ± 87.3			
α-Amylase	18 °C	269.9 ± 128.3	254.1 ± 91.3	225.3 ± 127.9	210.4 ± 96.6	***	ns	ns
	25 °C	804.9 ± 267.3	693.4 ± 426.7	507.7 ± 193.3	606.0 ± 235.8			
Lipase	18 °C	14.5 ± 4.1	14.0 ± 2.3	12.3 ± 3.0	13.6 ± 5.6	*	ns	ns
	25 °C	21.0 ± 5.9	16.9 ± 9.0	14.9 ± 9.0	15.8 ± 5.7			
Posterior gut								
Total Alkaline Protease	18 °C	337.1 ± 101.3 ab	405.9 ± 113.9 b	302.1 ± 162.4 ab	208.2 ± 100.6 a	***	ns	*
	25 °C	641.4 ± 166.8	543.5 ± 137.6	688.5 ± 129.7	603.6 ± 154.4			
α-Amylase	18 °C	293.8 ± 50.1	314.4 ± 115.0	222.7 ± 71.1	161.5 ± 83.7	***	ns	ns
	25 °C	1009.7 ± 262.3	910.6 ± 204.2	1011.3 ± 74.9	932.1 ± 240.5			
Lipase	18 °C	15.6 ± 6.4	16.3 ± 6.2	15.9 ± 10.1	13.4 ± 5.6	***	ns	ns
	25 °C	33.5 ± 7.7	24.9 ± 5.0	27.0 ± 4.1	30.1 ± 4.3			

Values presented as means ± standard deviation (n = 9).
*P < 0.05; ***P < 0.001; ns: not significant.
[a] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for diets within each temperature, and values in the same line with different letters are significantly different (P < 0.05).

Table 9
Gut histology of gilthead sea bream fed the experimental diets at two temperatures.

	Temperature	Diets			
		D0	D0.1	D0.25	D0.5
Anterior gut	18 °C	1.76 ± 0.15	1.76 ± 0.17	1.73 ± 0.15	1.73 ± 0.16
	25 °C	1.71 ± 0.17	1.69 ± 0.14	1.86 ± 0.16	1.86 ± 0.15
Posterior gut	18 °C	1.86 ± 0.47	1.85 ± 0.18	1.90 ± 0.20	1.81 ± 0.24
	25 °C	1.81 ± 0.12	1.72 ± 0.27	1.77 ± 0.25	1.84 ± 0.17

Values presented as means ± standard deviation (n = 9). Score from 1 to 5, with 5 indicating major alterations.
Mean scores were calculated by averaging the scores of the separate parameters evaluated (changes observed in mucosal folds height, width and cellularity of the lamina propria and submucosa, number of intraepithelial lymphocytes, nucleus position within the enterocytes and size and variation of enterocyte vacuolization).

reported to suppress lymphocytes proliferation [55]. In beluga (*Huso huso*), lymphocytes percentage was reduced in fish fed a 0.2% MOS diet [56]. Thus, the reduction of lymphocytes numbers observed in the present study may be related to an increased production of butyric acid in fish fed diets with 0.1% scFOS, but this requires to be confirmed.

Fish reared at 25 °C exhibited higher Hb content and RBC numbers compared to fish reared at 18 °C. This was not unexpected, since RBC are responsible for providing O_2 to the cells, and fish O_2 requirement increases while O_2 dissolved in water decreases with the increase of water temperature. Similar results were previously reported for rainbow trout (*Oncorhynchus mykiss*) exposed to simulated winter, spring, and summer conditions [57].

Plasma NO values are in line with those of RBC. Indeed, besides its immune function, NO also acts as a vasodilator, being produced at higher levels in fish reared at higher temperatures [58]. NO production was also affected by prebiotic incorporation, with higher values being observed in fish fed the 0.5% scFOS diet. In turbot, it was reported that *Lactococcus lactis* increased NO production in head kidney macrophage and serum, both *in vitro* and *in vivo*, respectively, possibly due to a synergistic effect between *L. lactis* and cytokines or other soluble factors [59]. Although higher LAB population in fish fed the 0.5% scFOS diet cannot be discarded, we were unable to detect it by the PCR-DGGE technique used in the present study.

At 25 °C, plasma total Ig was lower in fish fed the 0.5% scFOS

diet. Although, such a result was not observed on lymphocytes numbers, B-cells the ones responsible for Ig secretion [41,60], might change the Ig production rates, without changing total cell numbers. Regarding temperature effect, total Ig results are in line with lymphocytes results, being higher in fish reared at 18 °C, with fish fed control diet being not affected by the rearing temperature.

Though it is usually assumed that adaptive immune parameters tend to be suppressed at low temperatures [41], this is not a general rule. Indeed, in this study, fish reared at 18 °C and fed scFOS had higher total Ig values and lymphocyte counts than fish reared at 25 °C.

Monocytes and neutrophils are known to have strong phagocytosis ability and bactericidal and anti-protease activities [61,62]. In this study, monocytes and neutrophils numbers were higher in fish reared at 18 °C, but plasma bactericidal activity and NO production were higher in fish reared at 25 °C. In contrast, anti-protease activity was unaffected by water temperature. As in this study, Machado et al. [47] also observed that neutrophils and monocytes numbers may increase without showing increased humoral immune parameters, such as lysozyme and peroxidase activities.

In the present study, an effect of prebiotic incorporation on gut bacterial composition could not be detected. This contradicts results of other studies that reported FOS effects in growth and survival of beneficial bacteria present in fish gut [24,25,63]. Although PCR-DGGE is a useful technique to assess microbial community structures and ecological characteristics, allowing identification of bacteria that are not detected using culture dependent techniques [64], it is not as sensible as quantitative techniques such as FISH, qPCR, and next-generation sequencing. These quantitative techniques should therefore be preferred in future studies to overcome the semi-quantitative limitations associated with PCR-DGGE [64,65]. The apparent lack of scFOS effect could also be related with the high levels of fiber and oligosaccharides already present in the plant feedstuffs used in the experimental diets. For instance, soybean meal contains 4–5% oligosaccharides which may also have prebiotic-like properties, thus masking the effect of scFOS. Accordingly, also in gilthead sea bream, an absence of gut microbiota modulation was reported in fish fed a soy-rich diet supplemented with MOS, contrasting with gut microbiota modulation by MOS when fish were fed fish-meal-based diets [36].

Although several studies reported changes in gut microbiota communities in response to water temperature [12,39,66,67], such effect was not observed in the present study. The only detectable effect was an increase of similarity between DGGE profiles at 25 °C, indicating that higher temperatures might modulate gut bacteria population towards a higher similarity between samples. To the authors' knowledge there is only one study on the effects of temperature on prebiotics on fish gut microbiota modulation [12]. In contrast with present results, in that study it was shown that turbot fed scFOS presented higher gut bacteria richness and diversity at 20 °C than at 15 °C. There are other studies on the effects of temperature on fish gut microbiota, but used culture-dependent methods and are therefore difficult to compare with the culture-independent results presented in this study [39,66,67].

Regarding OTUs identity, the bacteria identified in the present study corresponded mostly to uncultured bacteria. Similar results were also observed in previous studies [8,27,68]. This is not surprising, since it is known that cultivable bacteria correspond to a small fraction of the total bacterial diversity that can be found associated with a complex and rich environment like the gut [68,69]. Nevertheless, a *Lactobacillus* sp., a genus of the LAB clade, was found among our samples. Although not dominant, LAB bacteria are often isolated from the gut of several fish species [39,70–72] and, interestingly, FOS was reported as supporting their growth and survival. The other two genus identified (*Cyanothece* and *Oceanobacillus*) corresponded to bacteria commonly found associated with marine environments [73,74].

The higher digestive enzymatic activity observed in fish reared at 25 °C correlates well with the higher feed intake, feed efficiency, and growth performance observed, and reported in Guerreiro et al. [11]. Although several studies reported increased digestive enzymatic activity or increased apparent nutrient digestibility in fish fed prebiotics [30–33,75,76], in the present study scFOS did not affect digestive enzymatic activity. This is in agreement with the lack of detectable scFOS effects in gut microbiota, as increased digestive enzymes activities were also suggested to be related to changes in gut microbiota. As in this study, other studies also failed to show increased digestive enzymatic activity in fish fed prebiotics supplemented diets [12,77–79]. Only TAP activity in the posterior gut was affected by temperature, however depending on fish diet, which reinforces the potential effect of temperature in prebiotic action.

Prebiotics, namely FOS, galactooligosaccharides (GOS), and MOS, have been reported to increase gut absorptive surface area due to increased microvilli density and height [26,35–37]. In the present study, however, no changes in gut histomorphology were noticed. This may also be related with the level of soybean meal in the experimental diets used in this study. Indeed, Bonaldo et al. [80] were able to observe minor histological alterations in the distal gut of gilthead sea bream fed diets including soybean meal at 30% but not at lower level (18%). Hence, whether deleterious effects of a more challenging diet could be alleviated by scFOS should be addressed in future studies. Also in gilthead sea bream, Dimitroglou et al. [36] found that dietary MOS had no effect on anterior gut mucosal folds morphology when observed by light microscopy. However, using electron microscopy it was shown that MOS positively affected gut ultrastructure. Since in the current study gut ultrastructure was not assessed, changes in gut morphology at ultrastructure level cannot also be discarded.

Results of this study indicate that gilthead sea bream innate immunity appears to be affected by rearing temperature, with particularly higher levels of circulating leucocytes at 18 °C, comparatively to 25 °C. On the contrary, digestive enzymes activities and gut microbiota similarity were higher in fish reared at 25 °C. Overall, dietary scFOS at the tested levels seem to have no major effects on gut microbiota composition, digestive enzymes activities, gut histomorphology and innate immune parameters. Thus, based on present data, it does not seem worthy of including scFOS in gilthead sea bream diets, at least at the tested levels. However, taking into account both the data of this study and that of Guerreiro et al. [11], it seems that rearing temperature might affect prebiotic outcomes, and this should be further exploited in other studies.

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Chapter 7
Improved glucose and lipid metabolism in European sea bass (*Dicentrarchus labrax*) fed short-chain fructooligosaccharides and xylooligosaccharides

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ABSTRACT

The effects of short-chain fructooligosaccharides (scFOS) and xylooligosaccharides (XOS) on growth, feed utilization and liver activity of key enzymes of glycolytic, gluconeogenic, and lipogenic pathways were studied in European sea bass juveniles. This is the first study about the effect of prebiotics on fish glucose metabolism and few and contradictory studies are available about prebiotic effect on lipid metabolism.
Fish were fed isoproteic (46%) and isolipidic (15%) diets based on fish meal (FM diets) or plant ingredients (PP diets; 30 FM:70 PP) as main protein sources. Four other diets were formulated similar to the control diets (PPC; FMC) but including 1% scFOS or 1% XOS (PPFOS, PPXOS, FMFOS and FMXOS diets). Growth performance was higher in fish fed PPXOS diet than PPC diet. No effect of dietary prebiotics on feed efficiency was noticed. Glucokinase activity was higher in fish fed FMFOS and FMXOS diets than FMC diet. Lipogenic enzyme activities (malic enzyme, fatty acid synthetase, glucose-6-phosphate dehydrogenase) were lower in fish fed diets including XOS than in the other groups. Glycolytic (glucokinase, pyruvate kinase) and lipogenic enzyme activities were higher, and gluconeogenic (fructose-1,6-bisphosphatase) enzyme activity was lower in fish fed FM diets than the PP diets. Overall, dietary XOS decreased lipogenesis, independently of dietary protein source, and improved growth performance in fish fed PP diets. In fish fed FM diets, XOS and scFOS increased glycolytic activity. XOS seemed to have good potential to be used as prebiotic in European sea bass.

1. Introduction

Prebiotics by definition are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of specific bacteria present in the gastrointestinal tract (GI), improving host health (Gibson and Roberfroid, 1995). Therefore prebiotics are selectively fermented by specific-health promoting bacteria such as *Lactobacillus* and *Bifidobacterium*, leading to a decrease of pathogenic bacterial species in the GI tract and/or to the production of fermentation end products, mainly short-chain fatty acids (SCFAs), which can modulate host glucose and lipid metabolism (Delzenne et al., 2008; Gibson and Roberfroid, 1995; Qiang et al., 2009; Roberfroid et al., 2010). Compared with humans and farm animals, less information is available concerning the effects of prebiotics on aquatic animals (Dimitroglou et al., 2011; Merrifield et al., 2010; Ringø et al., 2010).

Fructooligosaccharides (FOS) are among the most well-established prebiotics for use in aquafeeds (Ringø et al., 2010). FOS are produced on a commercial scale by two different processes, either through enzymatic hydrolysis of chicory-root inulin or from sucrose. FOS are composed by long linear chains of fructose units linked by β-(2–1) bonds attached to a terminal glucose unit. Short-chain fructooligosaccharides (scFOS) have a chemical composition similar to that of FOS but a degree of polymerization of only 1 to 5 glucose units (Bornet et al., 2002). FOS are fermented in the GI tract by beneficial bifidobacteria and other lactic-acid producing bacteria which possess β-fructosidase that hydrolyses β-(2–1) glycosidic bonds, an enzyme lacking in mammals and fish digestive tracts (Roberfroid and Slavin, 2000). In several fish species, it was reported that FOS and scFOS improved growth performance and feed efficiency, enhanced non-specific immune responses and disease resistance, improved gut function and morphological status, and increased health-promoting bacteria in the intestine (Anguiano et al., 2013; Hui-yuan et al., 2007; Ortiz et al., 2013; Soleimani et al., 2012; Zhou et al., 2009, 2010).

Xylooligosaccharide (XOS) is an emerging prebiotic gaining importance as functional ingredient in pharmaceutics, feed and food formulation (Aachary and Prapulla, 2011). Industrially, XOS is produced by chemical or enzymatic hydrolysis of xylan, which is

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the major component of lignocellulosic raw materials (Vázquez et al., 2000). Although the chemical structure of XOS depends on the xylan source, it generally consists of chains of xylose linked by β-(1–4) bonds, with a degree of polymerization ranging from 2 to 10. Studies in mammals showed that XOS promoted activity of beneficial intestinal bacteria, mainly *Bifidobacterium* species, leading to an increase of caeca SCFAs (Broekaert et al., 2011). These two effects are related with a number of health benefits, including improvement of bowel function, mineral absorption, lipid and glucose metabolism, immunomodulatory activity, reduction of colon cancer risk, and to antioxidant, anti-inflammatory and anti-microbial functions (Aachary and Prapulla, 2011; Broekaert et al., 2011). Up to now, only two studies were performed to evaluate XOS potential in fish (Li et al., 2008; Xu et al., 2009). In allogynogenetic crucian carp (*Carassius auratus gibelio*) growth performance and digestive enzyme activities were higher in fish fed 100 mg XOS kg^{-1} compared to the control diet (Xu et al., 2009). Dietary XOS incorporation at 400 mg kg^{-1} enhanced growth performance and nonspecific immunity of juvenile turbot (*Scophthalmus maximus*) (Li et al., 2008).

It has been reported that prebiotics, namely FOS and XOS, can alter glucose and lipid metabolism in mammals, depending on colonic fermentation process (Broekaert et al., 2011; Delzenne et al., 2008; Roberfroid et al., 2010). Thus, several studies indicated that FOS and XOS improved glucose tolerance, by lowering plasma glucose levels and enhancing insulin sensitivity (Gobinath et al., 2010; Respondek et al., 2011; Sheu et al., 2008; Shinoki and Hara, 2011), and reduced hepatic lipogenesis, serum and liver cholesterol, and triacylglycerides levels and increased serum HDL/LDL ratio (Fiordaliso et al., 1995; Kok et al., 1996; Sheu et al., 2008; Wang et al., 2011). However, the effect of prebiotics on lipid metabolism in fish was scarcely studied (Guerreiro et al., 2014; Torrecillas et al., 2011b) and no studies are available concerning prebiotic effects on glucose metabolism.

In contrast to probiotics, few studies on prebiotic effects were conducted in European sea bass (*Dicentrarchus labrax*), and focused only on the effect of mannanoligosaccharides (MOS) on the immune system and disease resistance (Torrecillas et al., 2007, 2011a,b, 2012, 2013). The aim of the present study was thus to assess the role of scFOS and XOS, incorporated into plant protein or fish meal based diets, on European sea bass glucose and lipid metabolism, through measurement of activities of key liver enzymes of glycolytic, gluconeogenic and lipogenic pathways.

2. Materials and methods

2.1. Diets

Two control diets were formulated to be isoproteic (46% crude protein) and isolipidic (15% crude lipid). One diet included fish meal (FM) as the main protein source (FMC diet) and the other diet included FM and plant ingredients (PP; soybean meal, wheat meal, wheat gluten and corn gluten) at a ratio of 30 FM:70 PP as protein sources (PPC diet). In both diets, cod liver oil was used as the main lipid source. Control diets also differ in terms of starch source. PPC diet contained approximately 13% of wheat starch and FMC diet contained approximately 20% of pregelatinized maize starch. In the experimental diets two commercial available prebiotics — scFOS (PROFEED Maxflow, Jefo, France) and XOS (Qingdao FTZ United International Inc., Quingdao, China) were added to the control diets at 1%, replacing α-cellulose (PPFOS, PPXOS, FMFOS and FMXOS diets). All dietary ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3 mm die. The pellets were then dried in an oven (40 °C) for 24 h and stored in plastic containers until used. The ingredients and proximate composition of the experimental diets are presented in Table 1.

Table 1
Ingredient composition and proximate analysis of the experimental diets.

	Diets					
	PPC	PPFOS	PPXOS	FMC	FMFOS	FMXOS
Ingredients (% dry weight basis)						
Fish meal[a]	15.6	15.6	15.6	59.2	59.2	59.2
Soluble fish protein concentrate[b]	5.0	5.0	5.0	5.0	5.0	5.0
Soybean meal[c]	25.0	25.0	25.0	–	–	–
Wheat meal[d]	18.3	18.3	18.3	–	–	–
Wheat gluten[e]	15.0	15.0	15.0	–	–	–
Corn gluten[f]	5.2	5.2	5.2	–	–	–
Cod liver oil	11.5	11.5	11.5	8.9	8.9	8.9
Pregelatinized maize starch[g]	–	–	–	22.4	22.4	22.4
Fructooligosaccharide[h]	–	1.0	–	–	1.0	–
Xylooligosaccharide[i]	–	–	1.0	–	–	1.0
Vitamin premix[j]	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix[k]	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5	0.5
α-Cellulose	1.0	–	–	1.0	–	–
Binder[l]	1.0	1.0	1.0	1.0	1.0	1.0
Proximate analyses (% dry weight basis)						
Dry matter	89.5	89.1	89.8	88.6	93	96.1
Crude protein	45.7	45.9	46	46.2	46.7	47.4
Crude fat	14.9	15.4	15.2	15.4	15	15.1
Ash	6.9	6.9	6.9	12.5	12.6	12.7
Starch	12.6	14.3	13.2	19.7	20.2	19.0
Gross energy (kJ g^{-1} DM)	22.9	22.7	23.5	21.1	20.9	21.2

DM, dry matter; CP, crude protein; GL, gross lipid.
[a] Inproquise, Madrid, Spain (CP: 70.1% DM; GL: 8.8% DM).
[b] Sopropèche G, France (CP: 79.4% DM; GL: 19.7% DM).
[c] Sorgal, S.A. Ovar, Portugal (CP: 50.5% DM; GL: 1.7% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 11.8% DM; GL: 1.9% DM).
[e] Sorgal, S.A. Ovar, Portugal (CP: 82.8% DM; GL: 1.9% DM).
[f] Sorgal, S.A. Ovar, Portugal (CP: 65.7% DM; GL: 3.5% DM).
[g] C-Gel Instant — 12016, Cerestar, Mechelen, Belgium.
[h] PROFEED Maxflow, Jefo, France.
[i] Qingdao FTZ United International Inc., Quingdao, China.
[j] Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); α-tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[k] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).
[l] Aquacube. Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium phosphate).

2.2. Animals and experimental conditions

The experiment was conducted according to the recommendations of the European Union Directive 2010/63/EU on the protection of animals for scientific purposes. The growth trial was performed at the Marine Zoology Station, Porto University, Portugal, in a thermoregulated recirculating water system equipped with 18 fibreglass tanks (100 L capacity) supplied with continuous flow of filtered seawater (6.0 L min^{-1}), temperature regulated to 25.0 ± 1.0 °C, salinity of 36.0 ± 1.0 g L^{-1} and dissolved oxygen kept near saturation (7.0 mg L^{-1}).

European sea bass juveniles were obtained from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain) and after transportation to the experimental facilities fish were submitted to a quarantine period of 15 days. During that period fish were fed with a commercial diet (48% protein, 11% lipids, 5% starch). Thereafter, 18 groups of 20 fish with an initial mean body weight of 60 ± 0.01 g were established and each diet randomly assigned to triplicate tanks. The trial lasted 7 weeks and during that period fish were fed by hand, 6 days a week, until visual satiation. Utmost care was taken to assure that all feed supplied was consumed. No mortality occurred during the growth trial.

2.3. Sampling

Fish in each tank were bulk-weighed at the beginning and at the end of the trial, after 1 day of feed deprivation. For that purpose, fish were slightly anaesthetised with 0.3 ml L^{-1} ethylene glycol monophenyl ether. After the final weighing fish continued to be fed for 3 more days to minimize manipulation stress and then 6 fish from each tank were sampled 6 h after the morning meal. Blood from 3 fish per tank was collected from the caudal vein with a heparinised syringe and immediately centrifuged at 10 000 $\times g$ for 10 min. Plasma aliquots were frozen at −80 °C until analysis of glucose, triacylglycerides, cholesterol and total lipids. Fish were then sacrificed with a sharp blow in the head and dissected on chilled trays. Livers were weighed for determination of hepatosomatic index (HSI) and then stored to quantify glycogen and lipid contents. Other 3 fish were sacrificed as above and livers collected for measurement of the activities of key enzymes of intermediate metabolism. After being collected livers were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

2.4. Proximate analysis of the diets

Chemical analyses of the diets were performed following the Association of Official Analytical Chemists methods (AOAC, 2000). Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA) and starch according to Beutler (1984).

2.5. Plasma metabolites and liver composition

Plasma glucose, cholesterol, triacylglycerides and total lipids were analysed using enzymatic colorimetric kits from Spinreact, Girona, Spain (glucose kit, code 1001191; cholesterol kit, code 1001091; triacylglycerides kit, code 1001312; total lipids kit, code 1001270). Hepatic glycogen and liver lipids were measured as described by Plummer (1987) and Folch et al. (1957), respectively.

2.6. Enzyme activity

In order to measure hexokinase (HK; EC 2.7.1.1), glucokinase (GK; EC 2.7.1.2), and L-type pyruvate kinase (PK; EC 2.7.1.40) activities, liver samples were homogenized (dilution 1:10) in ice-cold buffer (80 mM Tris-base; 5 mM EDTA; 2 mM dithiothreitol; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, pH 7.6). Homogenates were centrifuged at 900 $\times g$ for 10 min at 4 °C and the resultant supernatant was separated for HK/GK and PK activity measurements. The HK (low Km HKs) and GK (high Km HK or HK IV) activities were measured using 0.5 mM and 100 mM of glucose, respectively, as described previously (Panserat et al., 2000; Tranulis et al., 1996). To measure PK activities, the supernatant was centrifuged at 10 000 $\times g$ for 20 min at 4 °C and the resultant cytosolic fraction was used for enzyme activity measurements according to Foster and Moon (1985).

To measure the activity of fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11), liver samples were homogenized (dilution 1:10) in ice-cold buffer (20 mM Tris–HCl; 5 mM EDTA; 2 mM dithiothreitol; 0.24 M saccharose, pH 8). The homogenate was centrifuged at 900 $\times g$ for 10 min at 4 °C. Enzyme assays were performed on cytosolic fractions as previously described by Tranulis et al. (1996).

For glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), malic enzyme (ME; EC 1.1.1.40) and fatty acid synthetase (FAS; EC 2.3.1.38) activities, liver samples were homogenized (dilution 1:5) in ice-cold buffer (0.02 M Tris–HCl; 0.25 M sucrose; 2 mM EDTA; 0.1 M sodium fluoride; 0.5 mM phenyl methyl sulphonyl fluoride (PMSF); 0.01 M β-mercapto ethanol, pH 7.4) and the homogenate centrifuged at 30 000 $\times g$ for 20 min at 4 °C. G6PD activity was measured according to Bautista et al. (1988), ME activity according to Ochoa (1955) and FAS activity according to Chang et al. (1967) as modified by Chakrabarty and Leveille (1969).

Enzyme assays were carried out at 37 °C and the changes in absorbance were monitored to determine the enzyme activity using a microplate reader (ELx808; Bio-Tek Instruments, Winooski, Vermont, USA). The reagents used in the enzymatic analysis were purchased from Sigma-Aldrich (Sigma-Aldrich Química, S.L., Sintra, Portugal) and were of reagent grade.

All enzyme activities were expressed per mg of hepatic soluble protein (specific activity). Protein concentration was determined according to Bradford (1976) using a Sigma protein assay kit (ref. B6916) with bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol of substrate per min at assay temperature.

2.7. Statistical analysis

Results are presented as means with their standard deviations. Statistical analysis was done by two-way ANOVA using the SPSS 21.0 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were tested for normality by the Shapiro–Wilk test and homogeneity of variances by the Levene's test. When normality was not verified values were transformed prior to ANOVA. In the case of interaction between factors a one-way ANOVA analysis was also performed for prebiotics within each protein source. The probability level of 0.05 was used for rejection of the null hypothesis. Significant differences among groups were determined by the Tukey's multiple range test.

3. Results

In fish fed PP diets, but not on fish fed FM diets, growth performance was improved by XOS supplementation (Table 2). Protein source had no effect on fish growth performance ($P = 0.136$). Feed intake (FI) was affected by dietary protein source ($P = 0.000$) being higher in fish fed PP diets. In contrast, feed efficiency (FE) ($P = 0.000$) and protein efficiency ratio (PER) ($P = 0.000$) were higher in FM-fed fish. Prebiotic supplementation had no effect on FI, FE, and PER, independently of dietary protein source ($P > 0.05$).

Plasma glucose ($P = 0.000$) and cholesterol ($P = 0.000$) levels were higher in fish fed FM diets while plasma total lipids ($P = 0.001$) and triacylglycerides ($P = 0.000$) levels were higher in fish fed PP diets (Table 3). All plasma parameters measured were unaffected by prebiotic supplementation ($P > 0.05$).

Hepatosomatic index ($P = 0.000$) and liver glycogen content ($P = 0.000$) were higher in fish fed the FM diets (Table 3) while liver lipid content ($P = 0.307$) was not affected by dietary protein source. All above mentioned parameters were unaffected by dietary prebiotic supplementation ($P > 0.05$).

All measured liver enzymatic activities were affected by the dietary protein source (Table 4). HK, GK, PK, G6PD, ME (all with $P = 0.000$) and FAS ($P = 0.002$) activities were higher in fish fed the FM diets whereas FBPase ($P = 0.000$) activity was higher in fish fed PP diets. HK, PK and FBPase ($P > 0.05$) activities were unaffected by prebiotic supplementation. In contrast, G6PD ($P = 0.000$), ME ($P = 0.001$) and FAS ($P = 0.009$) activities were decreased in fish fed the XOS supplemented diets. In fish fed FM diets, but not the PP diets, GK activity was higher in the scFOS and XOS groups.

4. Discussion

Prebiotics were reported as growth promoters in some studies performed in fish (Dimitroglou et al., 2011; Merrifield et al., 2010; Ringø et al., 2010). The eventual positive effect of prebiotics on growth performance is dependent on prebiotic source, fish species, and other factors, including diet composition. In the present study, XOS improved

Table 2
Growth performance and feed utilization efficiency of European sea bass fed experimental diets[a].

	Diets					
	PPC	PPFOS	PPXOS	FMC	FMFOS	FMXOS
Final body weight (g)	98.4 ± 2.1[a]	101.0 ± 6.4[a,b]	108.5 ± 1.0[b]	107.0 ± 2.8	106.8 ± 1.1	103.6 ± 7.1
Weight gain (%IBW[†])	64.1 ± 3.5[a]	68.3 ± 10.7[a,b]	80.8 ± 1.7[b]	78.3 ± 4.6	78.0 ± 1.9	72.6 ± 11.7
Feed intake (g kg ABW^{-1}[§] day^{-1})	15.9 ± 0.5	16.7 ± 0.9	16.5 ± 0.3	14.3 ± 0.2	14.4 ± 0.6	15.0 ± 0.5
Feed efficiency[b]	0.66 ± 0.03	0.61 ± 0.03	0.69 ± 0.02	0.79 ± 0.05	0.80 ± 0.03	0.76 ± 0.02
Protein efficiency ratio[c]	1.44 ± 0.07	1.34 ± 0.07	1.47 ± 0.04	1.70 ± 0.11	1.72 ± 0.07	1.59 ± 0.05

Two-way ANOVA

	Variation source[d]		
	Protein	Prebiotic	Interaction
Final body weight (g)	0.136	0.410	0.038
Weight gain (%IBW)	0.135	0.408	0.038
Feed intake (g kg ABW^{-1} day^{-1}[§])	0.000	0.130	0.372
Feed efficiency[b]	0.000	0.744	0.039
Protein efficiency ratio[c]	0.000	0.531	0.026

[†] IBW: initial body weight.
[§] ABW: average body weight (initial body weight + final body weight) / 2.
[a] Mean values and standard deviation (±SD) are presented for each parameter (n = 3).
[b] FE: (wet weight gain / dry feed intake).
[c] PER: (wet weight gain / crude protein intake).
[d] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for prebiotics within each protein source and values in the same line with different superscript letters are significantly different ($P < 0.05$).

growth performance in fish fed PP diets but not in FM diets, and scFOS did not affect growth performance of the animals, independently of diet composition. The absence of XOS effect on growth performance of fish fed FM diets was possibly related to the high quality and digestibility of the diet, comparatively to the PP diet (Enes et al., 2006) which might have masked potential prebiotic effects. Improved growth with dietary XOS supplementation was previously reported by Li et al. (2008) in turbot and Xu et al. (2009) in allogynogenetic crucian carp. The lack of growth response due to dietary scFOS supplementation is also in agreement with previous studies in Atlantic salmon (*Salmo salar*) (Grisdale-Helland et al., 2008), red drum (*Sciaenops ocellatus*) (Buentello et al., 2010), and turbot (Guerreiro et al., 2014).

Previous studies also reported improved FE in fish fed diets supplemented with prebiotics, including a study with dietary XOS supplementation in turbot (Li et al., 2008). This higher FE was attributed to increased digestibility that could be related to increased exogenous microbial activity (Soleimani et al., 2012). In the present study, however no FE improvement was noticed due to prebiotic supplementation, independently of dietary protein source.

In mammals it was reported that FOS and XOS may alter glucose and lipid metabolism (Broekaert et al., 2011; Delzenne et al., 2008; Roberfroid et al., 2010). To the authors knowledge this is the first study in fish assessing the potential of prebiotics in modulating glucose metabolism. In fish fed FM diets, an increase of GK activity due to dietary

Table 3
Plasma glucose, cholesterol, triacylglycerides (mmol L^{-1}) and total lipids levels (g dL^{-1}) and hepatosomatic index (HSI), liver glycogen (g 100 g^{-1} liver) and lipids (g 100 g^{-1} liver) contents in European sea bass fed experimental diets[a].

	Diets					
	PPC	PPFOS	PPXOS	FMC	FMFOS	FMXOS
Plasma						
Glucose	5.4 ± 0.6	5.1 ± 1.6	5.3 ± 0.9	6.1 ± 1.4	7.0 ± 0.8	8.2 ± 3.4
Cholesterol	8.9 ± 0.5	8.9 ± 1.2	9.7 ± 1.3	11.9 ± 1.3	11.4 ± 0.6	11.6 ± 1.6
Total lipids	2.3 ± 0.3	2.2 ± 0.3	2.3 ± 0.2	1.9 ± 0.3	2.0 ± 0.2	2.1 ± 0.5
Triacylglycerides	17.8 ± 1.2	17.5 ± 2.1	17.3 ± 1.7	15.0 ± 2.5	15.5 ± 2.1	14.7 ± 1.9
Liver						
HSI	1.6 ± 0.2	1.6 ± 0.3	1.5 ± 0.3	2.2 ± 0.3	2.4 ± 0.2	2.5 ± 0.4
Glycogen	6.8 ± 2.1	5.6 ± 1.6	6.7 ± 1.9	8.0 ± 2.9	9.6 ± 1.9	9.8 ± 2.2
Lipids	9.4 ± 1.6	12.0 ± 1.3	9.7 ± 4.1	10.5 ± 1.0	11.4 ± 1.9	11.0 ± 1.6

Two-way ANOVA

	Variation source		
	Protein	Prebiotic	Interaction
Plasma			
Glucose	0.000	0.236	0.168
Cholesterol	0.000	0.390	0.340
Total lipids	0.001	0.595	0.726
Triacylglycerides	0.000	0.723	0.802
Liver			
HSI	0.000	0.559	0.132
Glycogen	0.000	0.480	0.128
Lipids	0.307	0.055	0.364

[a] Mean values and standard deviation (±SD) are presented for each parameter (n = 9).

Table 4
Hepatic glycolytic (hexokinase, HK, glucokinase, GK and pyruvate kinase, PK), gluconeogenic (fructose-1,6-bisphosphatase, FBPase) and lipogenic (glucose-6-phosphate dehydrogenase, G6PD, malic enzyme, ME and fatty acid synthetase, FAS) enzyme activities (mU mg protein^{-1}) in European sea bass fed experimental diets[a].

	Diets					
	PPC	PPFOS	PPXOS	FMC	FMFOS	FMXOS
HK	0.38 ± 0.18	0.42 ± 0.11	0.41 ± 0.15	0.64 ± 0.15	0.80 ± 0.16	0.62 ± 0.25
GK	2.3 ± 0.6	1.8 ± 0.5	1.7 ± 0.7	5.9 ± 1.7[a]	11.0 ± 2.7[b]	10.3 ± 2.2[b]
PK	81.4 ± 6.7	81.9 ± 3.5	80.8 ± 5.9	115.0 ± 15.6	112.1 ± 18.4	99.0 ± 8.5
FBPase	37.1 ± 5.8	34.2 ± 7.5	33.4 ± 9.0	27.5 ± 6.2	23.8 ± 4.6	24.9 ± 3.7
G6PD	146.7 ± 22.1	144.0 ± 27.3	120.2 ± 22.3	222.3 ± 22.3	232.7 ± 29.5	184.0 ± 25.7
ME	5.3 ± 0.9	5.5 ± 1.3	4.5 ± 0.7	7.3 ± 1.4	7.0 ± 1.3	5.6 ± 0.9
FAS	23.1 ± 2.7	22.1 ± 2.7	18.7 ± 4.0	24.5 ± 3.5	26.0 ± 3.4	22.8 ± 3.7

Two-way ANOVA

	Variation source[b]			Prebiotic		
	Protein	Prebiotic	Interaction	Control	FOS	XOS
HK	0.000	0.140	0.328			
GK	0.000	0.002	0.000			
PK	0.000	0.147	0.258			
FBPase	0.000	0.230	0.906			
G6PD	0.000	0.000	0.337	b	b	a
ME	0.000	0.001	0.426	b	b	a
FAS	0.002	0.009	0.405	b	b	a

[a] Mean values and standard deviation (±SD) are presented for each parameter (n = 9).
[b] Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for prebiotics within each protein source and values in the same line with different superscript letters are significantly different ($P < 0.05$).

prebiotic supplementation was observed while no such effect was evidenced in fish fed PP diets. These differences between diets are possibly related to the lower dietary starch content in the PP diets. In contrast to mammals, dietary prebiotic supplementation did not seem to have improved glucose tolerance, as plasma glucose levels were not different to that of the control groups. In line with the present results, no changes in plasma glucose levels were observed in juvenile beluga (*Huso huso*) fed with diets supplemented with oligofructose or inulin (Hoseinifar et al., 2011; Reza et al., 2009). Guerreiro et al. (2014) also reported no effect of scFOS on turbot glycaemia. Although not measured in the present study the SCFAs that are produced through prebiotic fermentation by gut microbiota, namely propionate, might explain the GK activity enhancement observed in fish fed FM diets. Thus, in isolated rat hepatocytes propionate stimulated glycolysis whereas the opposite was observed with acetate and butyrate (Anderson and Bridges, 1984). The enhancement of glycolysis observed with propionate was related with a decrease of hepatic citrate concentrations which is a metabolic inhibitor of phosphofructokinase (Blair et al., 1973). Thus, further studies, with SCFAs measurements are needed to understand the mechanism by which these end-fermentation products might affect GK activity. In mammals, besides stimulating glycolysis propionate also decreased gluconeogenesis via inhibition of pyruvate carboxylase, which catalyses the conversion of pyruvate to oxaloacetate (Anderson and Bridges, 1984; Blair et al., 1973). In contrast, acetate and butyrate increased glucose production from lactate in rat isolated hepatocytes (Anderson and Bridges, 1984). Present data on FBPase activity pointed to an absence of prebiotic effect on gluconeogenesis. On the other hand, in fish fed FM diets FBPase activity was lower than in fish fed PP diets, which had lower starch levels. Such a result is interesting, as previous studies with European sea bass have reported no effect of dietary carbohydrate level on hepatic FBPase activities Enes et al., 2006; Moreira et al., 2008).

In mammals, propionate also stimulated the production of the intestinal hormone glucagon-like peptide 1 (GLP-1), which in turn stimulated insulin secretion leading to an increase of liver glycogen synthesis and a decrease in plasma glucose levels (Cani et al., 2005; Delzenne et al., 2007; Frost et al., 2003). It is unlikely that such effects occurred in European sea bass as no effect of prebiotics on hepatic glycogen deposition and glycaemia was noticed in the present study.

As observed for the glycolytic enzymes activities, lipogenic enzymes activities were higher in fish fed FM diets than the fed PP diets, indicating that lipogenesis was stimulated by the higher dietary starch levels presented in FM diets. This was also previously observed in this species (Dias et al., 1998; Enes et al., 2010). Curiously, this higher lipogenesis is not reflected in liver lipid deposition or in plasma total lipids and triacylglycerides levels. Only plasma cholesterol levels were in line with the higher lipogenic enzyme activities recorded in fish fed FM diets. However, this might be related to the presence of soybean meal in the PP diets, as it is known that soybean components, such as phytosteroids have a hypocholesterolemic effect in fish (Gómez-Requeni et al., 2004; Kaushik et al., 1995, 2004), including European sea bass (Couto et al., in press).

In mammals, several studies reported a reduction of hepatic lipogenesis, plasma cholesterol and triacylglycerides levels due to dietary prebiotic supplementation (Causey et al., 2000; Fiordaliso et al., 1995; Gobinath et al., 2010; Kok et al., 1996; Letexier et al., 2003; Sheu et al., 2008; Wang et al., 2011). Accordingly, in this study dietary XOS supplementation also decreased the activity of G6PD, ME and FAS in fish fed both PP and FM diets. Similar results were observed by Torrecillas et al. (2011b) in European sea bass fed diets with MOS. In the present study, scFOS at a 1% incorporation level had no effect on lipogenic enzyme activities. This may be related to the dietary inclusion level, as Guerreiro et al. (2014) observed lower hepatic ME activity in turbot juveniles fed diets incorporating scFOS at 2%.

The lower hepatic lipogenesis due to dietary XOS supplementation could be related, as happened with GK, to the colonic production of SCFAs generated through XOS fermentation by fish gut microbiota (Delzenne et al., 2002, 2008; Teitelbaum, 2009). Thus, an increase of caecal SCFAs concentration namely acetate, propionate, butyrate and lactate was recorded in rats fed XOS supplemented diets (Campbell et al., 1997; Imaizumi et al., 1991). However, the modulation of lipid metabolism by the main SCFAs produced, acetate and propionate, is converse. Thus, whereas acetate is a lipogenic substrate, propionate is a competitive inhibitor of the entrance of acetate into liver cells (Delzenne et al., 2008; Teitelbaum, 2009). Propionate was also reported to inhibit lipid synthesis in rat hepatocytes (Nishina and Freedland, 1990; Wright et al., 1990). Moreover, acetate supplied in the diet of

diabetic mice at a dose of 0.3% activates liver AMP kinase, an enzyme related with the inhibition of lipogenesis (Sakakibara et al., 2006). In fact, in an in vitro study with hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) of around 200 g, incubation with GroBiotic®-A, MOS, and galactooligosaccharide (GOS) tends to produce lower acetate levels but higher butyrate levels at 48 h when compared to the diet alone (Burr et al., 2010). Of the SCFAs produced, acetate was the one produced in higher concentration after 24 h in inoculated samples, however after 48 h the concentration was reduced (Burr et al., 2010). On the other hand, in in vitro studies with rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), incubation with various saccharides lead the microbiota to produce higher levels of butyric and propionic acid (Kihara and Sakata, 2001, 2002). In in vivo studies, for instance in Siberian sturgeon (*Acipenser baerii*) fed arabinoxylan-oligosaccharides (AXOS) the production of acetate and butyrate was increased while propionate was produced in very low levels and similar to control diet (Geraylou et al., 2012, 2013).

Overall, it seems that the pattern of prebiotic fermentation by gut microbiota and the ratio of acetate to propionate reaching the liver will condition the potential lipid-lowering properties of prebiotics, moreover that ratio may change depending on the fish species. Therefore, in future studies the determination of SCFAs production should be measured in order to validate the hypotheses that in fish as in mammals the glucose and lipid metabolism might be mediated by bacteria SCFAs production. Even though XOS decreased hepatic lipogenesis, plasma total lipids, cholesterol and triacylglycerides, as well as liver lipid content, were unaffected by dietary prebiotic supplementation, which is contrary to what has been observed in mammals, where a reduction of these parameters is usually detected (Gobinath et al., 2010; Sheu et al., 2008; Wang et al., 2011).

In conclusion, diet supplementation with XOS improved growth performance in fish fed PP diets but not the FM diets. Independently of dietary protein source, supplementation with XOS decreased lipogenesis. In fish fed FM diets, XOS and scFOS increased glycolytic activity. Fish fed FM diets had increased glycolysis and lipogenesis and decreased gluconeogenesis, which seems to be related with the higher dietary starch content of these diets. Overall, use of XOS as prebiotic for European sea bass seems to have good potential and requires further investigation.

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Chapter 8
Gut morphology and hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles fed plant feedstuffs or fishmeal-based diets supplemented with short-chain fructo-oligosaccharides and xylo-oligosaccharides

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Gut morphology and hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles fed plant feedstuffs or fishmeal-based diets supplemented with short-chain fructo-oligosaccharides and xylo-oligosaccharides

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Abstract

The effects of short-chain fructo-oligosaccharides (scFOS) and xylo-oligosaccharides (XOS) on gut morphology and hepatic oxidative status were studied in European sea bass juveniles weighing 60 g. Fish were fed diets including fishmeal (FM diets) or plant feedstuffs (PF diets; 30 FM:70 PF) as main protein sources (control diets). Four other diets were formulated similar to the control diets but including 1 % scFOS or 1 % XOS. At the end of the trial, fish fed PF-based diets presented histomorphological alterations in the distal intestine, whereas only transient alterations were observed in the pyloric caeca. Comparatively to fish fed FM-based diets, fish fed PF diets had higher liver lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities, and lower glutathione peroxidase, glutathione reductase and glucose 6-phosphate dehydrogenase activities. In fish fed the PF diets, prebiotic supplementation decreased SOD activity and XOS supplementation further decreased CAT activity. In fish fed the FM diets, XOS supplementation promoted a reduction of all antioxidant enzyme activities. Overall, dietary XOS and scFOS supplementation had only minor effects on gut morphology or LPO levels. However, dietary XOS reduced antioxidant enzymatic activity in both PF and FM diets, which indicate a positive effect on reduction of hepatic reactive oxygen species production.

Key words: Antioxidant defences: European sea bass: Fish health: Intestinal histology: Plant feedstuffs: Prebiotics

To promote a sustainable aquaculture production, alternatives to fishmeal (FM) and fish oil are needed, as these commodities are still major protein and lipid sources in commercial aqua-feeds for carnivorous species[1]. Plant feedstuffs (PF) are nowadays the more available alternatives to FM, and overcome problems associated with fish by-products such as organic and inorganic contaminants, shortage of supply and net effect of demand-and-supply economics. However, PF usually contain anti-nutritional factors that may limit their use in aquafeeds[1,2].

Prebiotics are defined as ingredients that are not digested by the host but that are fermented by bacteria present in the gut, therefore promoting bacteria growth and/or activity[3]. Some of the beneficial effects of prebiotics in fish are improved growth rate, feed efficiency (FE), feed digestibility, fish survival, immunological status and resistance to bacterial and viral diseases[4–7]. Fructo-oligosaccharides (FOS) have already been evaluated as prebiotic in fish, but xylo-oligosaccharides (XOS) remain barely studied[4–7]. FOS are composed by short and medium chains of β-D-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages attached to a terminal glucose unit[6]. Short-chain fructo-oligosaccharides (scFOS) have a chemical composition similar to that of FOS but a smaller degree of polymerisation (between 1 and 5[8]). XOS are composed of xylose-based oligomers varying in the degree of polymerisation. Most of these oligomers consist of ester-linked phenolic acids such as ferulic, coumaric and caffeic acids[9,10].

Abbreviations: CAT, catalase; DI, distal intestine; FM, fishmeal; FMC diet, FM control diet; FOS, fructo-oligosaccharides; G6PD, glucose 6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; LPO, lipid peroxidation; MOS, mannan-oligosaccharides; PC, pyloric caeca; PF, plant feedstuffs; ROS, reactive oxygen species; SBM, soyabean meal; scFOS, short-chain fructo-oligosaccharides; SOD, superoxide dismutase; XOS, xylo-oligosaccharides.

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In fish fed diets containing plant ingredients, supplementation with prebiotics, namely mannan-oligosaccharides (MOS), *trans*-galacto-oligosaccharides (GOS) and FOS, improved intestinal morphology, by increasing gut absorptive area, microvilli density and height, and villi structure complexity[11–14]. In rats fed XOS-supplemented diets, total intestine weight increased relative to body weight, indicating augmented epithelial cell proliferation[15]. Up to now, there are, however, no studies accessing XOS effects on fish intestinal morphology.

PF and prebiotics were both reported to affect reactive oxygen species (ROS) generation in fish, decreasing oxidative damage and/or increasing antioxidant potential[16–21]. Prebiotics such as inulin were even reported as antioxidants, with ROS scavenging ability[22,23]. FOS was already reported as having antioxidant activity in fish, although mechanisms beyond that effect were not yet clarified[21,24]. XOS has ferulic acid in its composition, which was reported as having a very strong antioxidant activity[25], and studies in mammals showed that XOS decreased lipid peroxidation (LPO) and modulated antioxidant enzymatic activity[26–28]. However, XOS antioxidant potential has not yet been assessed in fish.

Thus, the present work aimed to evaluate the effect of dietary scFOS and XOS supplementation in gut morphology and hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) fed FM- or PF-based diets. This will contribute to elucidate the potential of prebiotics to mitigate some of the negative effects on fish health of using PF-based diets.

Methods

Diets

Two control diets were formulated to be isoproteic (46 % crude protein) and isolipidic (15 % crude lipid). One diet included FM as the main protein source (FM control diet: FMC diet) and the other diet included FM and PF (soyabean meal (SBM), wheat meal, wheat gluten and maize gluten) to provide a protein source ratio of 30 FM:70 PF (PF control diet: PFC diet). In both diets, cod liver oil was the main lipid source. Four other diets were formulated identical to the controls but including 1 % commercial prebiotics: scFOS (PROFEED Maxflow; Jefo) or XOS (Qingdao FTZ United International Inc.), replacing α-cellulose (diets PFFOS, PFXOS, FMFOS and FMXOS). All dietary ingredients were finely ground, well mixed and dry extruded in a laboratory pellet mill (California Pellet Mill) through a 3-mm die. The pellets were then dried in an oven (40°C) for 24 h and stored in plastic containers until use. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Animals and experimental conditions

The experiment was directed by FELASA category C certified scientists, and all procedures were conducted according to the European Union Directive 2010/63/EU on the protection of animals for scientific purposes. The growth trial was performed at the Marine Zoology Station, Porto University, Portugal, in a thermoregulated recirculating water system equipped with fibreglass tanks (100 litre capacity) supplied with continuous flow of filtered seawater (6·0 l/min), temperature regulated to 25·0 (SEM 1·0)°C, salinity of 36·0 (SEM 1·0 g/l and dissolved oxygen kept near saturation (7·0 mg/l).

European sea bass juveniles (*D. labrax*) were obtained from a commercial fish farm (Maresa S.A.) and after transportation to the experimental facilities fish were submitted to a quarantine period of 15 d. During that period fish were fed with a commercial diet (48 % protein, 11 % lipids, 5 % starch; A. Coelho & Castro Lda). Thereafter, eighteen groups of twenty fish with an initial mean body weight of 60 g were established and each diet was randomly assigned to triplicate tanks. The trial lasted 7 weeks, and during that period fish were fed by hand, 6 d/week, until visual satiation. No mortality occurred during the trial.

Sampling

After 7 and 15 d of the start of the feeding trial and at the end of the trial, three fish from each tank were randomly selected 2 h after the morning meal and euthanised with a sharp blow to the head. Fish were dissected on chilled trays and the digestive tract was freed from the adjacent adipose and connective tissues. Two pyloric caeca (PC) and a section of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) were sampled for histological evaluation. The samples were rinsed in PBS, carefully blotted dry with a paper towel, immediately fixed in phosphate-buffered formalin (4 %, pH 7·4) for 24 h and subsequently transferred to ethanol (70 %) until further processing. At the end of the trial, livers were also sampled from the same fish, immediately frozen in liquid N_2 and stored at −80°C until measurement of enzymes activities and LPO levels.

Proximate analysis of the diets

Chemical analyses of the diets were performed following the Association of Official Analytical Chemists methods[29]. Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 1261; PARR Instruments) and starch content was analysed according to Beutler[30].

Histological processing and morphological evaluation

PC and DI samples were processed and sectioned using standard histological techniques, and sections were stained with haematoxylin–eosin. Blind evaluation of histological preparations was performed with particular attention given to any inflammatory changes[31,32], namely shortening, widening and fusion of intestinal folds (FH), changes in enterocytes nucleous (ENT) and supranuclear absorptive vacuolisation (SNV), connective tissue hyperplasia in the lamina propria (LP) and submucosa (SM), and infiltration of inflammatory cells (intraepithelial leucocyte (IEL)). A continuous scale scoring system was used as described by Penn *et al.*[33], with the range of tissue scores set at 0–5. The overall value of histomorphological alterations was calculated by averaging scores of the separate parameters described above. Images were acquired with the Zen software (Blue edition; Zeiss).

Table 1. Ingredient composition and proximate analysis of the experimental diets

	Diets					
	PFC	PFFOS	PFXOS	FMC	FMFOS	FMXOS
Ingredients (% dry-weight basis)						
Fishmeal*	15·6	15·6	15·6	59·2	59·2	59·2
Soluble fish protein concentrate†	5·0	5·0	5·0	5·0	5·0	5·0
Soyabean meal‡	25·0	25·0	25·0	–	–	–
Wheat meal§	18·3	18·3	18·3	–	–	–
Wheat gluten‖	15·0	15·0	15·0	–	–	–
Maize gluten¶	5·2	5·2	5·2	–	–	–
Cod liver oil	11·5	11·5	11·5	8·9	8·9	8·9
Pregelatinised maize starch**	–	–	–	22·4	22·4	22·4
Fructo-oligosaccharide††	–	1·0	–	–	1·0	–
Xylo-oligosaccharide‡‡	–	–	1·0	–	–	1·0
Vitamin premix§§	1·0	1·0	1·0	1·0	1·0	1·0
Mineral premix‖‖	1·0	1·0	1·0	1·0	1·0	1·0
Choline chloride (50 %)	0·5	0·5	0·5	0·5	0·5	0·5
α-Cellulose	1·0	–	–	1·0	–	–
Binder¶¶	1·0	1·0	1·0	1·0	1·0	1·0
Proximate analyses (% dry-weight basis)						
DM	89·5	89·1	89·8	88·6	93	96·1
CP	45·7	45·9	46	46·2	46·7	47·4
Crude fat	14·9	15·4	15·2	15·4	15	15·1
Ash	6·9	6·9	6·9	12·5	12·6	12·7
Starch	12·6	14·3	13·2	19·7	20·2	19
Gross energy (kJ/g DM)	22·9	22·7	23·5	21·1	20·9	21·2

PFC, plant feedstuff control diet; PFFOS, plant feedstuff fructo-oligosaccharides; PFXOS, plant feedstuff xylo-oligosaccharides; FMC, fishmeal control diet; FMFOS, fishmeal fructo-oligosaccharides; FMXOS, fishmeal xylo-oligosaccharides; CP, crude protein; GL, gross lipid.
* Inproquise (CP: 70·1 % DM; GL: 8·8 % DM).
† Sopropèche G (CP: 79·4 % DM; GL: 19·7 % DM).
‡ Sorgal, S.A. (CP: 50·5 % DM; GL: 1·7 % DM).
§ Sorgal, S.A. (CP: 11·8 % DM; GL: 1·9 % DM).
‖ Sorgal, S.A. (CP: 82·8 % DM; GL: 1·9 % DM).
¶ Sorgal, S.A. (CP: 65·7 % DM; GL: 3·5 % DM).
** C-Gel Instant – 12016; Cerestar.
†† PROFEED Maxflow; Jefo.
‡‡ Qingdao FTZ United International Inc.
§§ Vitamins (mg/kg diet): retinol, 6.19; cholecalciferol, 0.04; *α*-tocopherol, 35; menadion sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0·02; biotin, 1·5; ascorbyl monophosphate, 50; inositol, 400.
‖‖ Minerals (mg/kg diet): cobalt sulphate, 1·91; copper sulphate, 19·6; iron sulphate, 200; sodium fluoride, 2·21; potassium iodide, 0·78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0·66; zinc oxide, 37·5; dibasic calcium phosphate, 5·93 (g/kg diet); potassium chloride, 1·15 (g/kg diet); sodium chloride, 0·44 (g/kg diet).
¶¶ Aquacube (guar gum, polymethyl carbamide, manioc starch blend, hydrated calcium phosphate) (Agil).

Enzyme activity

Liver samples were homogenised on ice in five volumes of ice-cold 100 mM-Tris-HCl buffer containing 0·1 mM-EDTA and 0·1 % (v/v) Triton X-100, pH 7·8. Homogenates were centrifuged at 30 000 ***g*** for 30 min at 4°C, and the resultant supernatants were separated in aliquots and stored at −80°C until use. All enzyme assays were carried out at 25°C in a microplate reader (ELx808; BioTek Instruments). The optimal substrate and protein concentrations for measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficients used for H_2O_2 and NADPH were 0·039 and 6·22 $mM^{-1} \times cm^{-1}$, respectively. Superoxide dismutase (SOD, *EC* 1.15.1.1), catalase (CAT, *EC* 1.11.1.6), glutathione peroxidase (GPX, *EC* 1.11.1.9), glutathione reductase (GR; *EC* 1.6.4.2) and glucose 6-phosphate dehydrogenase (G6PD, *EC* 1.1.1.49) activities were determined as described by Enes *et al.*[34]. Protein concentration in the homogenates was determined by the Bradford method[35] using the Sigma protein assay kit (ref. B6916) (Sigma-Aldrich Química, S.L.) with bovine serum albumin as a standard.

Enzyme activity was expressed as units (SOD, CAT) or milliunits (GPX, GR and G6PD) per mg of hepatic soluble protein. Except for SOD, one unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate/min under the assay conditions. One unit of SOD activity was defined as the amount of enzyme necessary to produce 50 % inhibition of the ferricytochrome c reduction rate.

Lipid peroxidation

Malondialdehyde (MDA) concentration was used as a marker of LPO level in the liver. In the presence of thiobarbituric acid, MDA reacts producing coloured thiobarbituric acid-reacting substances that were measured as described by Enes *et al.*[34]. Results were expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

Statistical analysis

Data analysis was done by two-way ANOVA using SPSS 21 software package (SPSS® Inc.). Previous to ANOVA, data were

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tested for normality and homogeneity (Shapiro–Wilk and Levene's tests, respectively) and when necessary transformed to achieve ANOVA assumptions. When significant interaction between factors were found, one-way ANOVA was performed within each protein source (PF and FM). Significant differences among means were determined with the Tukey's multiple range test. Histological data were neither normal nor homogeneous and could not be normalised, and they were analysed by the Kruskal–Wallis non-parametric test followed by pairwise comparisons. For all data, the probability level for rejection of the null hypotheses was 0·05.

Results

Growth performance, feed efficiency and feed intake

Growth performance was not the aim of this study, and it was presented in detail elsewhere[36]. Briefly, protein source had no effect on fish growth, but feed intake (FI) was higher and FE was lower in fish fed PF diets (Table 2). Prebiotic supplementation had no effect on growth of fish fed FM but XOS improved growth performance in fish fed PF diets. Prebiotic supplementation had no effect on FI and FE, independently of dietary protein source.

Gut morphology

Mean scores of the average of the separate parameters (FH, LP, SM, IEL, ENT and SNV) evaluated in PC and DI during the course of the trial are presented in Table 3.

No histomorphological alterations were observed with time in fish fed the FM-based diets. In fish fed the PF-based diets, histomorphological alterations with time were significant in the PC, but not in the DI.

In the PC, replacement of FM by PF resulted in an increase of mean scores of the evaluated parameters, and this increase was significantly higher after 15 d of feeding the experimental diets. Addition of FOS and XOS to the diets had no effect on PC morphology relatively to the respective control diets. Further, at 15 d of feeding, the mean scores of the evaluated parameters in fish fed the PFXOS diet was not different from that of the FMC diet. Histomorphological alterations observed in PF groups were transient, as by the end of the trial mean scores returned to values similar to the FMC diet. Overall, the most relevant observation in PC was the presence of enterocyte swelling (Fig. 1) in fish fed diets containing PF, which resulted in higher ENT and SNV scores in the mentioned groups (for both characteristics: $P<0{\cdot}001$, $P<0{\cdot}001$ and $P=0{\cdot}001$ at time points 7, 15 and final, respectively). To allow a better visualisation of the data, a graph with the several histological parameters evaluated in the PC was made (Fig. 2(a)). As no major differences were observed regarding the effects of prebiotics, and to simplify visualisation, data were pooled and presented only as FM and PF groups.

PF diets induced more pronounced effects in DI histomorphology, as denoted by the higher mean scores. Replacement of FM by PF resulted in an increase of mean scores of the evaluated parameters in the DI at all sampling days. The addition of prebiotics to PF diets did not ameliorate the negative effects in DI histomorphology.

Table 2. Growth performance and feed utilisation efficiency of European sea bass fed the experimental diets (Mean values with their pooled standard errors; *n* 3)[36]

								Two-way ANOVA*		
Diets	PFC	PFFOS	PFXOS	FMC	FMFOS	FMXOS	Pooled SEM	Protein	Prebiotic	Interaction
Final body weight (g)	98·4^{a}	101·0^{a,b}	108·5^{b}	107·0	106·8	103·6	1·20	0·136	0·410	0·038
Feed intake (g/kg ABW per d)†	15·9	16·7	16·5	14·3	14·4	15·0	0·26	<0·001	0·130	0·372
FE‡	0·66	0·61	0·68	0·79	0·80	0·76	0·02	<0·001	0·744	0·039

^{a,b} Mean values within the same line with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* Two-way ANOVA: if interaction was significant, one-way ANOVA was performed for prebiotics within each protein source.
† Average body weight (ABW): initial body weight + final body weight/2.
‡ Feed efficiency (FE): wet weight gain/dry feed intake

Table 3. Intestinal histology (pyloric caeca (PC) and distal intestine (DI)) of European sea bass fed the experimental diets for 7 and 15 d and by the end of the trial (final)*
(Mean values with their pooled standard errors; *n* 9)

Sections	Time	PFC	PFFOS	PFXOS	FMC	FMFOS	FMXOS	Pooled SEM
PC	7 d	^{B}1·83	^{B}1·62	^{B}1·51	1·14	1·18	1·26	0·28
	15 d	^{A}1·43^{b,c}	^{B}1·80^{c}	^{A,B}1·36^{a,b,c}	1·17^{a}	1·21^{a,b}	1·20^{a,b}	0·24
	Final	^{A}1·36^{b}	^{A}1·32^{a,b}	^{A}1·23^{a,b}	1·26^{a,b}	1·19^{a}	1·18^{a,b}	0·07
DI	7 d	2·13^{c}	2·09^{c}	2·11^{c}	1·12^{a}	1·55^{a,b,c}	1·42^{a,b}	0·42
	15 d	2·13^{c,d}	2·32^{d}	2·06^{b,c,d}	1·10^{a}	1·43^{a,b,c}	1·37^{a,b}	0·50
	Final	2·02^{b,c}	2·21^{c}	2·00^{b,c}	1·16^{a}	1·54^{a,b}	1·50^{a,b}	0·40

PFC, plant feedstuff control diet; PFFOS, plant feedstuff fructo-oligosaccharides; PFXOS, plant feedstuff xylo-oligosaccharides; FMC, fishmeal control diet; FMFOS, fishmeal fructo-oligosaccharides; FMXOS, fishmeal xylo-oligosaccharides.
^{A,B,a,b,c,d} Mean values with unlike superscript lowercase letters stand for statistical differences across dietary groups within each sampling day; mean values with unlike superscript uppercase letters stand for statistical differences for each parameter with time, as determined by the Kruskal–Wallis all pairwise comparisons ($P<0{\cdot}05$).
* Mean scores were calculated by averaging the scores of the separate parameters evaluated (shortening, widening and fusion of intestinal folds, changes in enterocytes nucleous and supranuclear absorptive vacuolization, connective tissue hyperplasia in the lamina propria and submucosa, and infiltration of inflammatory cells). Score from 0 to 5, with 5 indicating major alterations.

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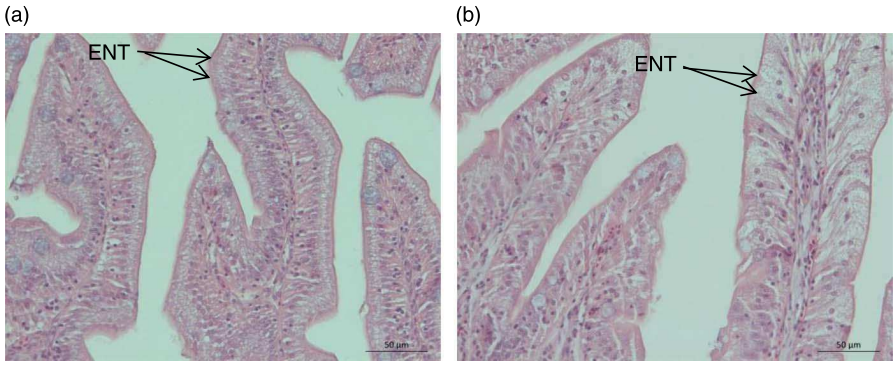

Fig. 1. Detail of enterocyte nucleousswelling (ENT) in the pyloric caeca of fish fed fishmeal xylo-oligosaccharides (a) and plant feedstuff xylo-oligosaccharides (b) at time point 7 d. Scale bar: 50 μm; haematoxylin–eosin staining.

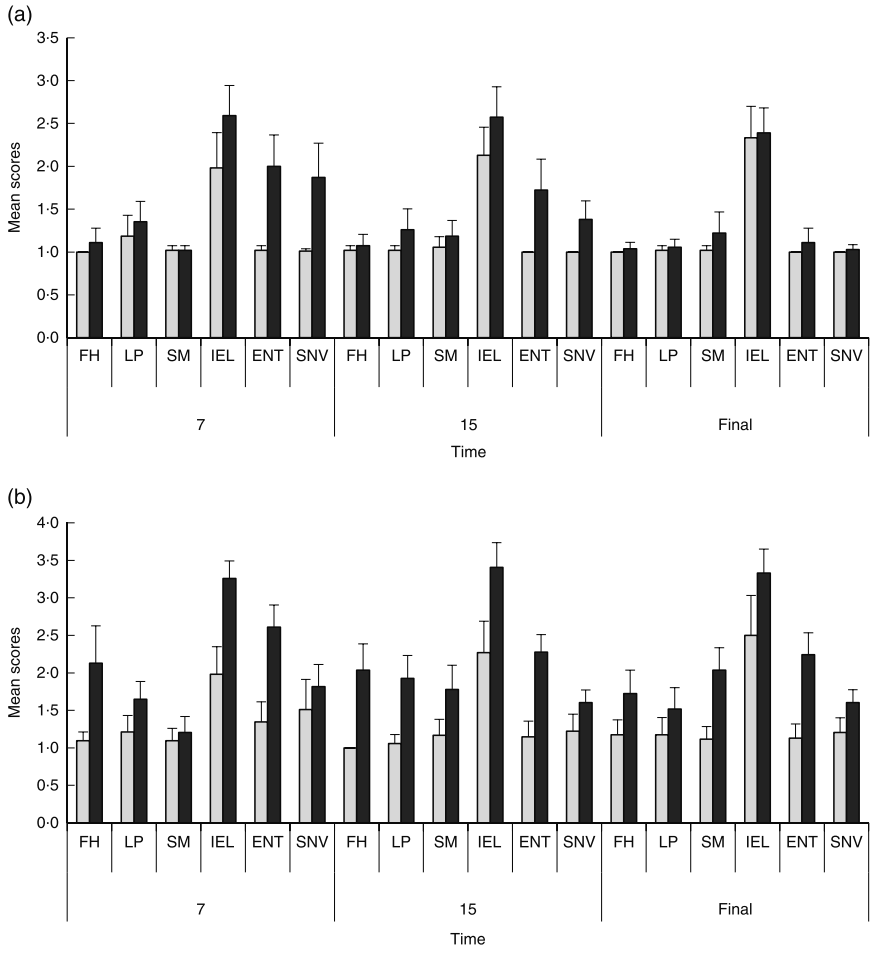

Fig. 2. Mean scores, with their standard errors for pyloric caeca (a) and distal intestine (b) of FH (shortening, widening and fusion of intestinal folds), LP (connective tissue hyperplasia in the lamina propria), SM (connective tissue hyperplasia in the submucosa), IEL (infiltration of inflammatory cells), ENT (changes in enterocytes nucleous) and SNV (changes in supranuclear absorptive vacuolisation). As no major differences were observed regarding the effects of prebiotics, and to simplify visualisation, data were pooled and presented only as FM (□) and PF (■) groups. Data are separated for sea bass fed the experimental diets for 7 and 15 d and by the end of the trial (final). Score from 0 to 5, with 5 indicating major alterations.

The most pronounced histological alterations observed in the DI of fish fed PF diets (Fig. 3) were as follows: decreased height of the mucosal folds ($P<0{\cdot}001$, $P<0{\cdot}001$ and $P=0{\cdot}004$ in time points 7, 15 and final, respectively), increased number of IEL ($P<0{\cdot}001$, $P<0{\cdot}001$ and $P<0{\cdot}001$ in time points 7, 15 and final, respectively) and abnormal size variation of SNV ($P=0{\cdot}013$, $P=0{\cdot}002$ and $P=0{\cdot}001$ in time points 7, 15 and final, respectively). As explained above, a graph was also made for

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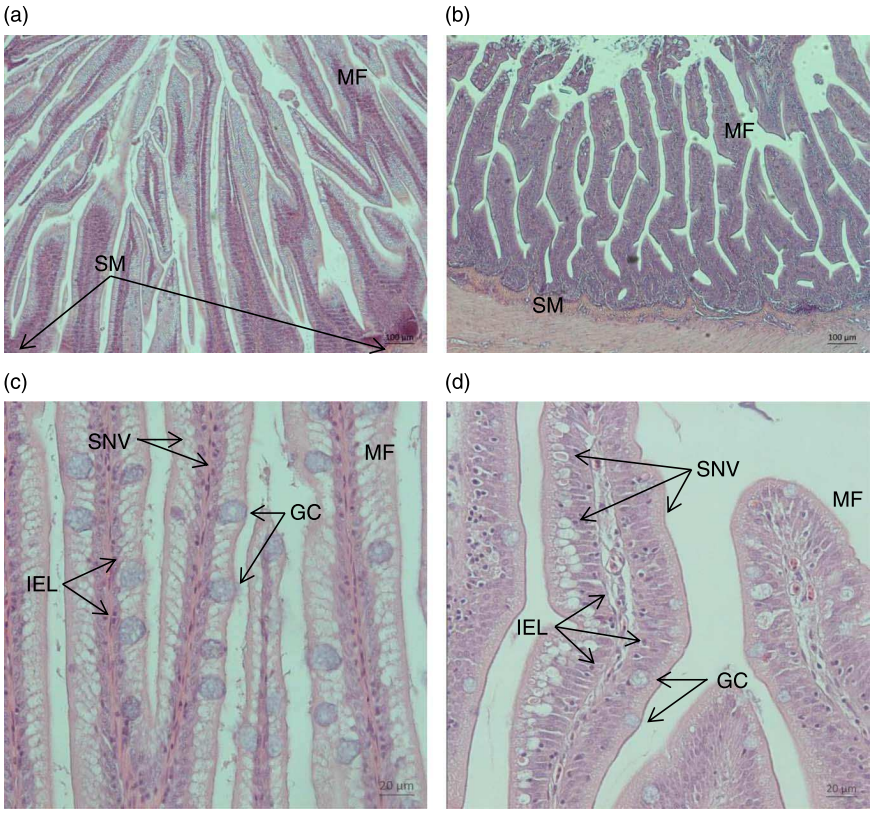

Fig. 3. Distal intestine alterations in fish fed FM control diet (a, c) and PF control diet (PFC) (b, d), showing decreased fold height, increased width of the lamina propria and intraepithelial leucocytes (IEL), as well as abnormal enterocyte vacuolisation and nucleous position in PFC group at time point 15 d. MF, mucosal fold; SM, submucosa; GC, goblet cell; SNV, supranuclear vacuole. Scale bars: 100 µm (a, b); 20 µm (c, d); haematoxylin–eosin staining.

Table 4. Specific activities of hepatic antioxidant enzymes and lipid peroxidation (LPO) levels of European sea bass fed the experimental diets* (Mean values with their pooled standard errors; *n* 9)

								Two-way ANOVA†		
Diets	PFC	PFFOS	PFXOS	FMC	FMFOS	FMXOS	Pooled SEM	Protein	Prebiotic	Interaction
SOD	219^{c}	190^{b}	165^{a}	168^{a,b}	182^{b}	149^{a}	3·7	<0·001	<0·001	0·001
CAT	191	203	173	145	148	127	4·7	<0·001	0·004	0·822
GPX	65	74	75	101^{b}	85^{a,b}	71^{a}	2·3	<0·001	0·065	<0·001
GR	1·91	2·34	2·11	3·30^{b}	3·21^{b}	2·32^{a}	0·08	<0·001	<0·001	<0·001
G6PD	171^{a}	211^{b}	173^{a}	289^{b}	320^{c}	240^{a}	8·3	<0·001	<0·001	0·006
LPO	12·62	10·89	12·16	9·25	9·38	9·36	0·25	<0·001	0·123	0·072

PFC, plant feedstuff control diet; PFFOS, plant feedstuff fructo-oligosaccharides; PFXOS, plant feedstuff xylo-oligosaccharides; FMC, fishmeal control diet; FMFOS, fishmeal fructo-oligosaccharides; FMXOS, fishmeal xylo-oligosaccharides; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; G6PD, glucose 6-phosphate dehydrogenase.

^{a,b,c} Mean values within the same line with unlike superscript letters are significantly different ($P < 0·05$).

* SOD and CAT are expressed as U/mg protein. GPX, GR and G6PD are expressed as mU/mg protein. LPO is expressed as nmol malondialdehyde/g tissue.

† Two-way ANOVA: for CAT, two-way ANOVA results showed significant differences among control, fructo-oligosaccharides and xylo-oligosaccharides, regardless of protein source, being Tukey's test results as b-b-a, respectively. For other parameters, if interaction was significant, one-way ANOVA was performed for prebiotics within each protein source.

the DI portion (Fig. 2(b)). Opposite to what was observed in PC, histological alterations did not recede and were similar at days 7, 15 and by the end of the trial.

Antioxidant enzymatic activity and lipid peroxidation

Antioxidant enzymatic activity and LPO levels at the end of the trial are present in Table 4. Protein source affected all parameters analysed. LPO levels, SOD and CAT activities were higher, whereas GPX, GR and G6PD activities were lower in fish fed PF diets than the FM diets. LPO level and GPX activity were not affected by prebiotic incorporation, whereas CAT activity was decreased by XOS supplementation.

In fish fed PF diets, both scFOS and XOS decreased SOD activity, whereas in fish fed FM diets XOS led to a reduction of SOD, GPX, GR and G6PD enzymatic activities. Compared with the control groups, scFOS increased G6PD activity in both PF and FM diets.

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Discussion

A replacement of 95 % of FM by PF in diets for European sea bass was already accomplished without affecting fish growth, diets digestibility or voluntary FI[37]. However, in that study authors did not perform intestinal histomorphology observations. However, it is well known that the anti-nutritional factors presented in PF, namely those present in SBM, can cause moderate and severe enteritis in fish[31,38–41]. In the present study, although no overt inflammation was observed, fish fed PF diets presented alterations in the DI histomorphology when compared with fish fed FMC diets. Such an effect may be related to the inclusion of 25 % of SBM in the PF diets, as it was previously observed in Atlantic salmon (*Salmo salar*)[39,40]. However, it was previously reported in European sea bass that inclusion of 30 % SBM had no significant consequences in the DI histomorphology[42]. Thus, the DI modifications observed in the present study were more likely a consequence of the combination of different PF, and thus different anti-nutrients, in the PF diets. Accordingly, Couto *et al.*[43] fed European sea bass with two purified soyabean anti-nutrients, saponins and phytosterols, and observed no severe effects on fish gut histology, supporting the fact that the inflammation observed in the present study could be because of interaction/cumulative effects of anti-nutrients and PF sources.

In this study, the majority of permanent gut histological alterations were observed on the DI portion. A similar observation was reported by van den Ingh *et al.*[38] in salmon fed diets in which FM was partially replaced by 30 % full-fat SBM. The presence of inflammation only in the DI was related to the higher sensibility of this intestinal portion to PF anti-nutrients. In fact, the same was already reported in another study with salmon[44] and in European sea bass[43].

With regard to the PC section, although at 15 d fish fed PFC diets presented higher mean scores for the evaluated parameters than fish fed the FMC diet, at the end of the trial no histomorphological differences between the two groups were found. These results indicate that fish were able to adapt to the high dietary PF levels, reversing the initial histomorphological alterations. Such recovery from previous histological alterations because of diet changes has already been reported for European sea bass[43] and common carp (*Cyprinus carpio*)[45]. Urán *et al.*[45] hypothesised that this recovery was related to the omnivorous feeding habits of carp, making it prone to adapt to diets with high PF levels. This is not the case for sea bass, as it is a carnivorous species and thus does not feed on PF in nature. However, the present results indicate that European sea bass has higher tolerance for PF than salmon, which is also a carnivorous species. Indeed, salmon seems to be particularly intolerant to high levels of dietary PF. Actually, even rainbow trout (*Oncorhynchus mykiss*), which is also a carnivorous species that belongs to the same family as salmon, exhibits higher tolerance for SBM anti-nutritional factors[46].

Although prebiotics such as MOS, GOS and FOS have been reported as increasing gut absorptive surface area and microvilli density and height in fish fed plant-based diets[11–14], in the present study no histomorphological alterations were noticed in fish fed the PF diets. Other studies similar to the present one also reported no differences at optical microscopy level in the intestinal morphology of fish fed diets containing PF and prebiotics[11,47,48]. Dimitroglou *et al.*[12] fed gilthead sea bream (*Sparus aurata*) FM- and SBM-based diets supplemented with MOS, and also observed that MOS had no effect on the mucosal folds morphology of the anterior intestine. However, MOS appeared to improve the absorptive surface area in the posterior intestine of fish fed the FM diet, as denoted by higher perimeter of the intestinal lumen. However, using electron microscopy techniques, it became evident that MOS affected both anterior and posterior intestine at the ultrastructural level in fish fed FM- and SBM-based diets. As we did not assess the intestinal ultrastructure of fish intestine, it cannot be ruled out that histological alterations may have occurred at this level.

Differences between studies on the effects of prebiotics in gut histomorphology may also be related to prebiotic type, dosage, species assessed and age, rearing conditions, differences in gut microbiota or methodological approaches. For instance, Dimitroglou *et al.*[11] showed that gut histology of fish fed the same prebiotic (MOS) and diet may change in the same species depending on fish age. Authors observed improvements in gut morphology, such as increased absorptive surface, microvilli density and length, of subadult rainbow trout fed MOS, whereas no effects were observed in juveniles.

It was previously reported in rainbow trout, Atlantic salmon, gilthead sea bream and European sea bass that PF can reduce oxidative damage in fish[16,17,20]. This may be linked to PF components with strong antioxidant activity, namely phenolic compounds, flavonoids, α-tocopherol or astaxanthin[17,20].

Although the PF used in the present study are rich sources of flavonoids (SBM) and phenolic compounds, mostly in the form of ferulic acid (maize and wheat meal)[49,50], no improved antioxidant effect was observed in the PF diets. Indeed, fish fed PF diets presented higher hepatic LPO level than fish fed FM diets. Increased LPO occurs when ROS production is higher than ROS removal. SOD, which catalyses the dismutation of the superoxide anion (O_2^-) to molecular oxygen and H_2O_2, is the first enzyme responding to the presence of oxygen radicals, preventing the radical chain reaction initiated by O_2^-. In the present study, hepatic SOD activity was higher in fish fed PF diets, indicating that there was a rise of O_2^- generation in fish fed PF diets. Hepatic CAT activity was also increased, whereas hepatic GPX activity was lower in fish fed PF diets, indicating that CAT was the major route for reducing H_2O_2 to molecular oxygen and water. The different behaviour of the two peroxidation reduction routes may be related to the overall concentration of H_2O_2 generated, as it is known that CAT is more active when H_2O_2 production is high, whereas GPX is induced by low H_2O_2 levels[51]. Such low H_2O_2 levels are probably present in fish fed FM-based diets, as in this group significantly lower SOD activities were noticed. GR has a role in the modulation of GPX activity, as it catalyses the NADPH-dependent regeneration of GSH from GSSG generated by GPX[51]. Thus, and in order to maintain the GSSG:GSH ratio, higher hepatic GR activity was observed in fish fed FM diets because of the high GPX activity observed in fish fed those diets. Hepatic G6PD was also increased in fish fed FM diets, as it is involved in NADPH regeneration, which is a coenzyme required for GR activity[52,53].

Although LPO levels were unaffected by dietary prebiotic incorporation, XOS supplementation seemed to contribute to a reduction of overall ROS production, as CAT activity was lower in fish fed both PFXOS and FMXOS, and activities of GPX, GR and G6PD were also reduced in fish fed the FMXOS diet. Comparison of the present results with other studies performed in fish was not possible, as to the authors' knowledge there are no studies available regarding the effect of XOS on antioxidant status. In type 2 diabetes mellitus rats, XOS also contributed to a reduction of CAT activity in erythrocyte samples, but not of SOD and GPX activities[26]. The lower effects of XOS on the antioxidant enzymatic activity observed in fish fed the PF diets might be related to soyabean oligosaccharides that may have acted as prebiotics and masked the XOS effects[54–56].

In mammals, prebiotics fermentation by gut microbiota leads to the production of SCFA, which have a role in oxidative stress modulation. In addition, butyrate, more than other SCFA, was reported as being related with a significant reduction in H_2O_2-induced DNA damage in rats and humans[57–59]. Although fish have a less abundant gut microbiota than mammals and lack a large bowel, it is known that fermentation by fish gut microbiota also produces SCFA[60]. Nonetheless, the reported antioxidant ability of XOS is most likely related to its composition, as most of the oligomers present in XOS consist of ester-linked phenolic acids such as ferulic, coumaric and caffeic acids[9,10,25]. Ferulic acid has potent antioxidant potential mainly because of its phenolic nucleus and extended side-chain conjugation that readily forms stabilised phenoxy radicals and terminate chain reactions[25]. In fact, ferulic acid scavenges superoxide anion radicals in a similar way to SOD[61].

FOS or a combination of FOS and probiotics were already reported as having antioxidant capabilities in several fish species[18,19,21,62,63]. Zhang *et al.*[62] explained this antioxidant potential of FOS by its bifidogenic effect and suggested that the antioxidant properties of FOS might help in the gut microbial defence mechanism, as FOS may help surpassing both exogenous and endogenous oxidative stress. Another possibility is that FOS might have a role in the translation and post-translational process of the antioxidant enzymes[21]. However, further investigation is needed to understand the mechanisms behind FOS antioxidant capabilities. In the present study, except for a decrease of SOD activity in fish fed the PFFOS diet, scFOS had little effect in European sea bass oxidative status.

In conclusion, PF-based diets increased LPO levels and had negative impacts in the histomorphology of DI compared with fish fed FM-based diets. Prebiotic incorporation in PF diets was not effective in counterbalancing the negative effects of PF diets in gut morphology. However, XOS incorporation in both PF and FM diets reduced antioxidant enzymatic activity, suggesting a role in the reduction in ROS production.

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A. O.-T. and P. E. designed and coordinated the study. I. G, A. C., A. P.-J. and P. E. were responsible for the data collection, analysis and statistical analysis. All authors contributed to the interpretation of the data. I. G. wrote the first draft of the manuscript and all authors critically reviewed and revised the manuscript.

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Chapter 9
Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*)

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Submitted

Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*)

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Keywords

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Galactooligosaccharides;
Glycolysis;
Lipogenesis;
Short-chain fructooligosaccharides;
Xylooligosaccharides.

Abstract

This study aimed at evaluating the effects of short-chain fructooligosaccharides (scFOS), xylooligosaccharides (XOS) and galactooligosaccharides (GOS) on growth performance, hepatic metabolism, gut microbiota and digestive enzymes activities of white sea bream juveniles. Fish with an initial weight of 53 g were fed during 12 weeks with diets including fish meal (FM) and plant feedstuffs (PF) (30FM:70PF; control diet) as main protein sources. Three other diets were formulated similar to the control diet but supplemented with 1% dietary scFOS (FOS diet), XOS (XOS diet) or GOS (GOS diet). Dietary prebiotics incorporation had no effect on growth or feed efficiency. Plasma triglycerides levels were lower in fish fed XOS than FOS and GOS diets. Malic enzyme activity was lower in fish fed XOS than FOS diet. XOS diet also led to lower FAS activity compared to FOS and control groups. Alanine aminotransferase activity was higher in fish fed XOS than control diet. No effect of prebiotics on the activity of key enzymes of the glycolytic pathway was observed. Fifteen days after the start of the trial an enhancement of total alkaline protease, trypsin and lipase activities was observed in fish fed prebiotic supplemented diets, but such effect was not noticed by the end of the trial. Gut bacterial operational taxonomic units, richness and diversity, and digestive enzyme activities were higher 15 days after the start of the trial than by the end of the trial. Overall, prebiotics led to a transient increase of digestive enzymes activities. Independently of the prebiotic tested, a relation between gut indices and digestive enzyme activities was observed. Dietary supplementation with XOS decreased lipogenesis. In conclusion, among the prebiotics tested, XOS seems the most promising prebiotic to be used in white sea bream juveniles diets.

1. Introduction

Functional ingredients, such as probiotics, prebiotics and immunostimulants, are reported to improve growth, feed efficiency, stress tolerance, diseases resistance and health status in fish, and thus are nowadays increasingly being used in the aquafeed industry (Oliva-Teles, 2012). Prebiotics are feed ingredients that are not digested by the host but selectively stimulate the growth or the activity of gut beneficial bacteria, which provide energy and metabolic substrates to the host through prebiotic fermentation (Gibson and Roberfroid, 1995). Several studies reported growth improvement when fish were fed prebiotics (Dimitroglou et al., 2011; Merrifield et al., 2010a; Ringø et al., 2010, 2014). This can be related with several factors, such as changes in gut microbiota abundance or diversity, improved digestive enzymatic activity, improved gut morphology, or modifications in intermediary metabolism (Anguiano et al., 2013; Guerreiro et al., 2015c; Hoseinifar et al., 2013; Soleimani et al., 2012).

Short-chain fructooligosaccharides (scFOS) are fructooligosaccharides (FOS) with low degree of polymerization, and together with xylooligosaccharides (XOS) and galactooligosaccharides (GOS) are prebiotics that have not been much studied in fish (Dimitroglou et al., 2011; Merrifield et al., 2010a; Ringø et al., 2010, 2014). In mammals, FOS, XOS and GOS were reported to improve glucose tolerance, by lowering plasma glucose levels and enhancing insulin sensitivity, and to reduce hepatic lipogenesis, plasma cholesterol and triglycerides levels (Fiordaliso et al., 1995; Gobinath et al., 2010; Sangwan et al., 2015; Shinoki and Hara, 2011; Vulevic et al., 2013; Wang et al., 2011). Prebiotic effects in fish intermediary metabolism are restricted to a few studies (Guerreiro et al., 2015a, b, c; Torrecilas et al., 2011, 2015). Feeding turbot (*Scophthalmus maximus*) with scFOS, and European sea bass (*Dicentrarchus labrax*) with XOS and mannanoligosaccharides (MOS), led to a decrease in the hepatic activity of key lipogenic enzymes (Guerreiro et al., 2015a, c; Torrecilas et al., 2011). An increase in glycolytic activity was also observed in European sea bass fed fish meal-based diets supplemented with scFOS and XOS (Guerreiro et al., 2015c). However, no relevant effects of scFOS and XOS on glucose, cholesterol or triglycerides plasmatic levels were reported (Guerreiro et al., 2015a, b, c).

In mammals, changes on glucose and lipid metabolism have been attributed to changes on gut microbiota composition and to short-chain fatty acids (SCFAs) produced in prebiotics fermentation (Delzenne et al., 2002; Gibson and Roberfroid, 1995; Sangwan et al., 2015; Teitelbaum, 2009). Gut microbiota modulation may also lead to modification of the digestive

enzymes secreted by bacteria, thus affecting nutrients digestion (Renjie et al., 2010; Soleimani et al., 2012; Wu et al., 2013; Xu et al., 2009).

Therefore, the aim of the present study was to determine the effect of scFOS, XOS and GOS on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes activities of white sea bream (*Diplodus sargus*) juveniles fed plant feedstuffs-based diets.

2. Material and Methods

2.1. Diets composition

A diet was formulated to include 18% lipid and 37% protein using fish meal (FM) and plant feedstuffs (PF) as protein sources (at circa 30:70 of protein from FM:PF) and fish oil as lipid source (control diet). Three other diets were formulated identical to the control but including 1% commercial prebiotics: scFOS (PROFEED Maxflow, Jefo, France), XOS or GOS (Qingdao FTZ United International Inc., Quingdao, China), replacing α-cellulose (diets FOS, XOS, and GOS, respectively). All diet ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 2.0 mm die. Pellets were dried in an oven at 40 °C for 48 h and stored in a freezer in airtight bags until use. Ingredients and proximate composition of the diets are presented in Table 1.

Chemical analyses of the diets were performed following Association of Official Analytical Chemists Methods (AOAC, 2000). Dietary starch content was determined according to Beutler (1984).

Table 1
Ingredients and proximate composition of the experimental diets.

	Diets			
	Control	FOS	XOS	GOS
Ingredients (% dry weight)				
Fish meal[a]	15.4	15.4	15.4	15.4
Corn gluten[b]	13.3	13.3	13.3	13.3
Wheat meal[c]	25.1	25.1	25.1	25.1
Soy meal[d]	25.0	25.0	25.0	25.0
Cod liver oil	15.1	15.1	15.1	15.1
Bicalcium phosphate[e]	1.6	1.6	1.6	1.6
Fructooligosaccharide[f]	-	1.0	-	-
Xylooligosaccharide[g]	-	-	1.0	-
Galactooligosaccharide[h]	-	-	-	1.0
α-cellulose[i]	1.0	-	-	-
Vitamin mix[j]	1.0	1.0	1.0	1.0
Mineral mix[k]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder (Aquacube)[l]	1.0	1.0	1.0	1.0
Proximate analysis (% dry weight)				
Dry matter (%)	90.7	88.4	91.7	88.8
Crude protein	37.2	37.7	36.9	37.3
Crude fat	18.7	18.6	18.4	18.2
Ash	8.1	7.9	8.7	8.1
Starch	16.1	15.5	16.2	16.5
Gross energy (kJ g^{-1})[m]	18.9	18.9	18.8	18.8

DM: dry matter; CP: crude protein; GL: gross lipid.
[a] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 71.2% DM; GL: 9.1% DM).
[b] Sorgal, S.A. Ovar, Portugal (CP: 67.4% DM; GL: 2.7% DM).
[c] Sorgal, S.A. Ovar, Portugal (CP: 10.9% DM; GL: 2.8% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 54.1% DM; GL: 1.9% DM)
[e] Premix, Portugal (Calcium: 24%; Total phosphorus: 18%).
[f] scFOS: PROFEED Maxflow, Jefo, France.
[g] Quingdao FTZ United International Inc., Quingdao, China.
[h] Quingdao FTZ United International Inc., Quingdao, China.
[i] Sigma-Aldrich, Sintra, Portugal.
[j] Vitamins (mg kg^{-1} diet): retinol acetate, 18000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[k] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).
[l] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).
[m] Gross energy calculated based on theoretical values (CP: 23.6 kJ g^{-1}; GL: 39.5 kJ g^{-1}; Carbohydrates: 17.2 kJ g^{-1})

2.2. Growth trial

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with white seabream (*Diplodus sargus*) juveniles obtained from IPMA, Olhão, Portugal. The trial was performed in recirculating water systems equipped with 12 cylindrical fiberglass tanks of 100 L water capacity and thermo-regulated to 23.0 ± 0.5 °C. Tanks were supplied with continuous flow of filtered seawater (2.5-3.5 L min^{-1}) of 35 ± 1 g L^{-1} salinity and dissolved oxygen was kept near saturation (7 mg L^{-1}). After a quarantine period of 1 month, fish were transferred to the experimental systems and adapted to the experimental conditions for 15 days. Thereafter, 19 white seabream with an initial mean body weight of 53 ± 0.03 g were distributed to each tank and the experimental diets randomly assigned to triplicate groups. The trial lasted 12 weeks, and during that period fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. The experiment was performed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

2.3. Sampling

Fish in each tank were bulk-weighed at the beginning and at the end of the trial, after 1 day of feed deprivation. For that purpose, fish were slightly anaesthetised with 0.3 ml L^{-1} ethylene glycol monophenyl ether. After the final weighing, fish continued to be fed for 3 more days to minimize manipulation stress and then 6 fish from each tank were sampled 4 h after the morning meal. Blood from 3 fish per tank was collected from the caudal vein with heparinised syringes and immediately centrifuged at 10 000 × g for 10 min. Plasma aliquots were frozen at -80 °C until plasma metabolites analysis. Fish were then sacrificed with a sharp blow in the head and dissected on chilled trays. Livers were weighed for determination of hepatosomatic index (HSI) and then stored at -80ºC until quantification of glycogen and lipid contents. Gut, without pyloric caeca, but with intestinal content was removed, immediately frozen in liquid nitrogen and then stored at -80 °C until measurement of digestive enzyme activities. The 3 other fish were sacrificed as described above and livers collected for measurement of the activities of key enzymes of intermediate metabolism. After collection, livers were snap frozen in liquid nitrogen and stored at -80 °C until analysis. Guts from two of these fish were sampled under aseptic conditions for collection of intestinal digesta and subsequent microbiota

characterization. Digesta was obtained by squeezing the entire gut. Samples were immediately frozen in liquid nitrogen and then stored at -80 °C until analysis. Besides final sampling, an intermediary sampling at day 15 after the beginning of the trial was also performed for digestive enzymatic determination and gut microbiota characterization, as described above.

2.4. Plasma metabolites and liver composition

Plasma glucose, cholesterol, triglycerides and total lipids were analysed using enzymatic colorimetric kits from Spinreact, Girona, Spain (glucose kit, code 1001191; cholesterol kit, code 1001091; triglycerides kit, code 1001312; total lipids kit, code 1001270). Hepatic glycogen and lipids content were measured as described by Plummer (1987) and Folch et al. (1957), respectively.

2.5. Intermediary metabolism enzyme activity

Hexokinase (HK; EC 2.7.1.1), glucokinase (GK; EC 2.7.1.2) and L-type pyruvate kinase (PK; EC 2.7.1.40) activities were determined as described in Guerreiro et al. (2015c). Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), fatty acid synthetase (FAS, EC 2.3.1.38), malic enzyme (ME, EC 1.1.1.40), glutamate dehydrogenase (GDH, EC 1.4.1.2), alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were assayed as described in Guerreiro et al. (2015a). Enzyme assays were carried out at 37 °C in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

All enzyme activities were expressed per mg of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme that catalysis the hydrolysis of 1 µmol min^{-1} of substrate at assay temperature (37 °C). Protein concentration was determined by the Bradford method (Bradford, 1976) using Sigma-Aldrich protein assay kit (ref. B6916) with bovine serum albumin as a standard.

2.6. Microbial diversity analysis

The samples of 2 fish per tank were pooled to reduce variation. DNA was extracted from 300 mg of gut contents following the method of Pitcher et al. (1989), after mechanical lysis (2x 30 seconds) in a BeadBug Microtube Homogenizer (Benchmark Scientific, USA) in 500 µl STE buffer (0.1M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 0.4 g of glass beads (Sigma G8772). Bacterial 16S rRNA gene fragments were amplified by touchdown PCR on a T100TM Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal), using oligonucleotide primers 16S-358F (which contained a GC clamp at the 5´end) and 16S-517R (Muyzer et al., 1993). Approximately 300 ng of each PCR product were resolved on 8% polyacrylamide gel composed by a denaturing gradient of 40 to 80% 7M urea/40% formamide. Electrophoresis was performed on a DCode™ universal mutation detection system (Bio-Rad Laboratories Lda., Amadora, Portugal), during 16 h at 60 °C, 65V in 1×TAE buffer. Gels were stained for 1 h with SYBR-Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific Inc.) and imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal). Distinct bands were excised from the gel and eluted in 20 µl ultrapure water prior to DNA re-amplification (Muyzer et al., 1993) using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov) (Macrogen Europe, Amsterdam, The Netherlands). Only sequences higher than 140 bp reads and 80–100% query coverage were considered a valid identification.

2.7. Digestive enzymes activity

Intestine samples were homogenized (dilution 1:4) in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA and 0.1% (v/v) TritonX-100, pH 7.8). Homogenates were centrifuged at 30 000 × g for 30 min at 4 °C and the resultant supernatants were kept in aliquots and stored at -80 °C until use. For each assay, supernatant was properly diluted in homogenized buffer to ensure optimum ratio between enzymes and subtract. All procedures were performed on ice. Enzyme assays were determined using a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China).

Total protease activity (TAP) was measured by the casein-hydrolysis method described by Walter (1984) and adapted by Hidalgo et al. (1999). The reaction mixture containing casein (1% w/v; 0.125 mL), buffer (0.1 M Tris-HCl, pH 9.0; 0.125 mL) and homogenate supernatant (0.05 mL) was incubated for 1 h at 37 °C. The reaction was stopped by addition of 0.3 mL trichloroacetic acid (TCA) (8% w/v) solution. After being kept for 1 h at 4 °C, samples were centrifuged at 1 800 × g for 10 min and the supernatant absorbance measured at 280 nm. A control blank for each sample was prepared adding the supernatant from the homogenates after the incubation period. Tyrosine solution was used as standard.

Trypsin (EC3.4.21.4) activity was measured according to Faulk et al. (2007), using Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (1 mM BAPNA) as substrate. Trypsin assay buffer contained 50 mM Trizma and 20 mM $CaCl_2$, pH 8.2. Production of p-nitroanaline (molar extinction coefficient, 8.8 $mM^{-1}cm^{-1}$) was monitored at 37 °C and followed at 410 nm.

α-Amylase (EC 3.2.1.1) activity was measured at 405 nm by the rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, 12.9 $mM^{-1}cm^{-1}$) at 37 °C using a commercial kit from Spinreact, Girona, Spain (ref. 41201).

Lipase (EC 3.1.1.3) activity was measured using a Spinreact (Girona, Spain) kit (ref. 1001274). The rate of methylresorufin (molar extinction coefficient, 60.65 $mM^{-1}cm^{-1}$) formation was quantified photometrically (580 nm; 37 °C) and it is proportional to the concentration of catalytic lipase present in the sample homogenate.

All enzyme activities were expressed as specific activity (mU per mg of soluble protein); one unit (U) of activity was defined as the µmol of product generated per minute. Protein concentrations were determined by the Bradford method (Bradford, 1976) using a Sigma-Aldrich (Química, S.L., Sintra, Portugal) protein assay kit (ref. B6916) with bovine serum albumin as the standard.

2.8. Statistical analysis

Data are presented as means ± standard deviation. Statistical analysis of the digestive enzymes activities was done by two-way ANOVA with sampling time and diets as fixed factors, using SPSS 22 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were tested for normality by the Shapiro-Wilk test, and for homogeneity by the Levene's test. When normality was not verified data was transformed (cholesterol, amylase and TAP were logarithm normalized) prior to ANOVA. In the case of interaction between factors a one-way ANOVA was performed for diets within each sampling time. The remaining data was analysed by one-way ANOVA. Significant differences among groups were determined by the Tukey's

multiple range test. The probability level of 0.05 was used for rejection of the null hypothesis.

DGGE (Denaturing Gradient Gel Electrophoresis) banding patterns were transformed into presence/absence matrices and band intensities measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories Lda., Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef's measure of richness, and species diversity was assessed by the Shannon–Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the unweighted pair groups method with arithmetic averages (UPGMA). Gut microbiota parameters were subjected to two-way ANOVA with diet and sampling time as fixed factors, as described above.

3. Results

Fish promptly accepted the experimental diets and during the trial mortality was very low (< 5%). Growth, feed intake, feed efficiency (FE) and protein efficiency ratio (PER) were unaffected by dietary prebiotic incorporation (Table 2).

Plasma glucose, cholesterol and total lipids were unaffected by diet composition. Plasma triglycerides were lower in fish fed XOS than in fish fed FOS and GOS diets (Table 3). HSI and liver lipids were unaffected by dietary prebiotic incorporation, whereas liver glycogen was lower in fish fed FOS than the control and XOS diets (Table 3).

Hepatic key glycolytic enzymes activities measured (HK, GK and PK) were unaffected by dietary prebiotics (Table 4). In contrast, lipogenic enzymes activities (G6PD, ME and FAS) were all affected by dietary prebiotic incorporation. G6PD activity was higher in fish fed FOS than the other diets. ME activity was lower in fish fed XOS than FOS diet. FAS activity was also lower

Table 2
Growth performance and feed utilization efficiency of white sea bream fed the experimental diets.

Diets	Control	FOS	XOS	GOS
Final body weight (g)	92.7 ± 1.7	95.2 ± 2.9	91.4 ± 1.1	91.1 ± 4.8
Mortality (%)[a]	0	0	4.8 ± 4.1	4.8 ± 4.1
Weight gain (%)[b]	71.2 ± 2.8	74.6 ± 4.8	68.3 ± 1.7	67.8 ± 9.1
Feed intake (g kg ABW^{-1}[§] day^{-1})	14.4 ± 0.5	14.1 ± 0.3	13.7 ± 0.4	13.6 ± 0.7
Feed efficiency[c]	0.46 ± 0.01	0.49 ± 0.01	0.49 ± 0.02	0.48 ± 0.04
Protein efficiency ratio[d]	1.25 ± 0.02	1.30 ± 0.03	1.34 ± 0.05	1.28 ± 0.10

Values presented as means ± standard deviation (n=3). No significant differences were noticed between dietary treatments (P>0.05).
[§]ABW: average body weight (initial body weight + final body weight)/2
[a]Mortality: 100*(number of dead fish /number of initial fish)
[b]Weight gain (%): 100 *(final body weight - initial body weight)/ initial body weight
[c]Feed efficiency (FE): (wet weight gain/dry feed intake)
[d]Protein efficiency ratio (PER): (wet weight gain/crude protein intake)

Table 3
Plasmatic glucose (mg dL^{-1}), cholesterol, triglycerides and total lipids (g dL^{-1}), hepatosomatic index and liver composition (wet weight basis) of white sea bream fed the experimental diets.

Diets	Control	FOS	XOS	GOS
Plasma				
Glucose	87.8 ± 9.1	87.7 ± 7.6	84.0 ± 7.6	88.9 ± 10.0
Cholesterol	0.41 ± 0.07	0.37 ± 0.09	0.33 ± 0.05	0.34 ± 0.08
Triglycerides	0.54 ± 0.08^{ab}	0.63 ± 0.07^{b}	0.43 ± 0.10^{a}	0.63 ± 0.20^{b}
Total lipids	2.15 ± 0.37	2.07 ± 0.53	1.73 ± 0.38	2.09 ± 0.42
Liver				
HSI (%)[a]	1.20 ± 0.10	1.10 ± 0.16	1.31 ± 0.41	1.17 ± 0.16
Lipid (%)	6.15 ± 0.89	5.31 ± 0.78	6.20 ± 1.09	6.37 ± 1.00
Glycogen (%)	10.54 ± 0.90^{b}	7.24 ± 1.78^{a}	9.10 ± 0.98^{b}	8.88 ± 1.54^{ab}

Values presented as means ± standard deviation (n=9). Values in the same line with different letters are significantly different (P<0.05).
[a]Hepatosomatic index: 100*(liver weight/body weight)

Table 4
Specific activities (mU mg protein^{-1}) of hepatic glycolytic (hexokinase, HK, glucokinase, GK and pyruvate kinase, PK), lipogenic (glucose-6-phosphate dehydrogenase, G6PD, malic enzyme, ME and fatty acid synthetase, FAS) and amino acid catabolic enzymes (glutamate dehydrogenase, GDH, alanine aminotransferase, ALAT, aspartate aminotransferase, ASAT) in white sea bream fed the experimental diets.

Diets	Control	FOS	XOS	GOS
HK	0.32 ± 0.11	0.35 ± 0.08	0.42 ± 0.15	0.39 ± 0.10
GK	16.2 ± 4.1	12.3 ± 2.3	14.5 ± 3.1	14.1 ± 2.4
PK	31.6 ± 8.1	32.9 ± 2.9	35.4 ± 7.6	31.3 ± 5.8
G6PD	92.6 ± 16.5^{a}	113.6 ± 22.4^{b}	82.1 ± 10.9^{a}	79.6 ± 7.8^{a}
ME	6.24 ± 0.56^{ab}	6.58 ± 1.0^{b}	5.10 ± 1.11^{a}	5.96 ± 1.10^{ab}
FAS	4.91 ± 1.29^{b}	5.72 ± 1.38^{b}	2.76 ± 0.97^{a}	4.25 ± 0.96^{ab}
GDH	47.2 ± 6.1	52.2 ± 5.0	48.9 ± 6.6	52.3 ± 12.3
ASAT	609.8 ± 97.9	601.3 ± 33.5	581.6 ± 113.8	584.9 ± 87.6
ALAT	272.6 ± 47.2^{a}	332.6 ± 77.3^{ab}	357.3 ± 66.9^{b}	293.8 ± 35.5^{ab}

Values presented as means ± standard deviation (n=9). Values in the same line with different letters are significantly different (P<0.05).

in fish fed XOS than the control and FOS diets (Table 4). Within the enzymes involved in amino acid catabolism, GDH and ASAT were unaffected by dietary prebiotics, while ALAT was higher in fish fed XOS than the control diet (Table 4).

The Bray–Curtis dendrogram and the analysis of the hypervariable V3 16S rDNA DGGE fingerprints (Fig. 1) revealed that white sea bream gut bacterial communities maintain a similarity higher than 50%, independently of the dietary regime or the sampling time. Nevertheless, at 15 days more OTUs were observed. This correlates with the lower microbial richness and diversity obtained at the end of the trial (Table 5). However, the similarity index was not affected by sampling time. Dietary incorporation of prebiotics had no effect on the average number of OTUs, microbial richness, diversity and similarity (Table 5).

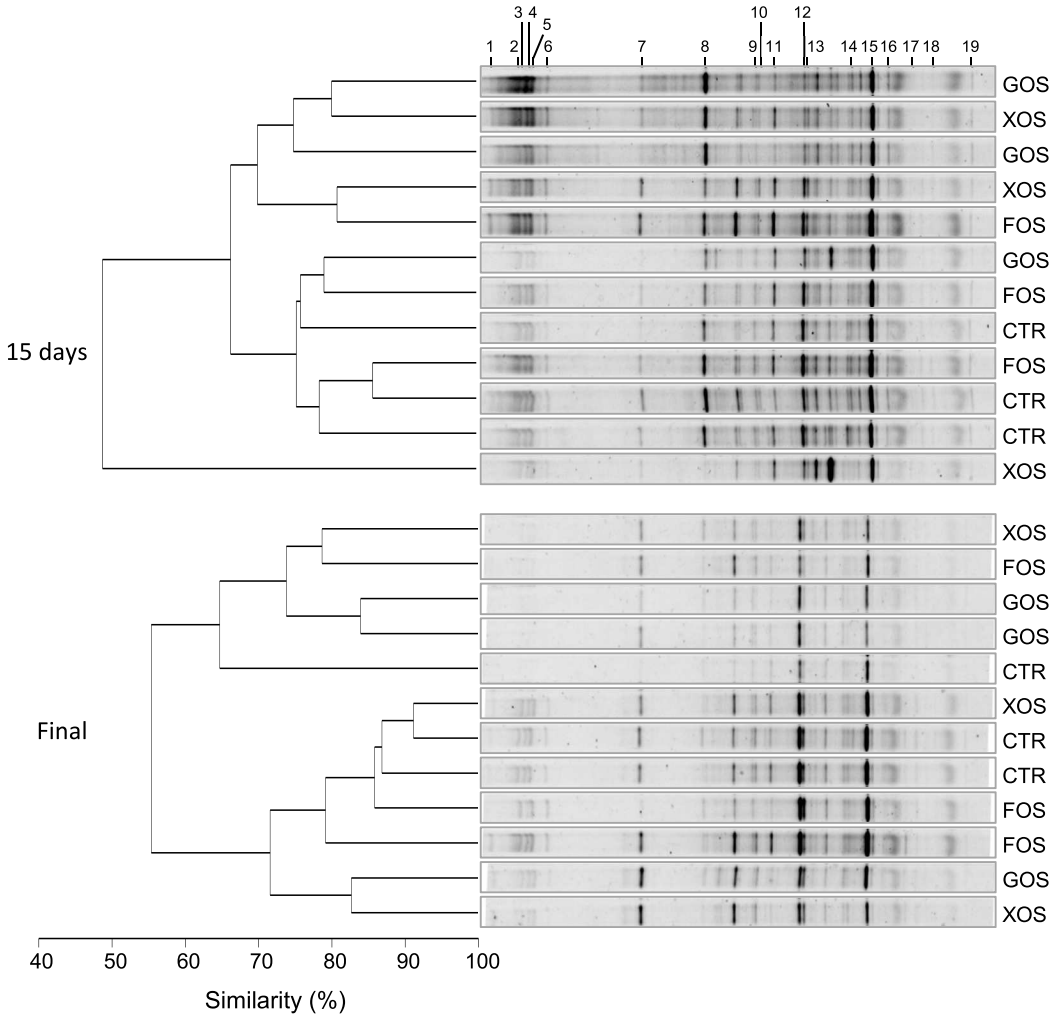

Figure 1. Dendrogram and PCR-DGGE fingerprints of the gut microbiota of white sea bream fed the experimental diets for 15 days and at the end of the trial. Numbers on top of the figure (1-19) indicate bands excised for sequence analysis.
CTR: control diet; FOS: short-chain fructooligosaccharides diet; XOS: xylooligosaccharides diet; GOS: galactooligosaccharides diet.

Table 5
Ecological parameters obtained from PCR-DGGE fingerprints of gut microbiota of white sea bream fed the experimental diets for 15 days and at the end of the trial.

Time	15 days				Final			
Diets	Control	FOS	XOS	GOS	Control	FOS	XOS	GOS
OTUs[a]	24.0 ± 1.0	26.7 ± 2.1	25.3 ± 8.6	26.3 ± 7.0	19.3 ± 8.1	19.7 ± 7.0	19.7 ± 4.2	16.0 ± 3.6
Richness[b]	1.42 ± 0.05	1.56 ± 0.11	1.49 ± 0.50	1.55 ± 0.39	1.15 ± 0.48	1.17 ± 0.40	1.19 ± 0.24	0.97 ± 0.21
Diversity[c]	3.08 ± 0.06	3.16 ± 0.10	3.07 ± 0.43	3.15 ± 0.30	2.75 ± 0.47	2.79 ± 0.33	2.87 ± 0.20	2.67 ± 0.19
SIMPER Similarity (%)[d]	76.9 ± 2.2	72.9 ± 4.6	55.4 ± 18.3	66.9 ± 8.2	56.4 ± 27.6	61.8 ± 12.8	72.4 ± 2.7	68.6 ± 13.5
Two-way ANOVA								
Variation source	Time	Diets	Interaction					
OTUs[a]	*	ns	ns					
Richness[b]	*	ns	ns					
Diversity[c]	*	ns	ns					
SIMPER Similarity (%)[d]	ns	ns	ns					

Values presented as means ± standard deviation (n = 3 per treatment pooled from 6 fish).
[a]OTUs: average number of operational taxonomic units.
[b]Margalef species richness: d=(S-1)/log(N).
[c]Shannons diversity index: H'=-∑(pi(lnpi)).
[d]SIMPER: similarity percentage within group replicates.
Two-way ANOVA: * $P < 0.05$; ns: not significant.

Sequence analysis from the DGGE bands (Table 6) showed that the majority of the dominant allochthonous bacteria detected were most closely related to uncultured bacteria previously isolated from the environment, plants or other animals. Four OTUs were most closely related to *Lactobacillus* sp. and other three to *Pseudomonas* sp.

The identified OTUs could be found in all treatments, although with different intensities, being less pronounced at the end of the trial than at 15 days. OTUs 17 and 18 were only visible at 15 days. Six OTUs were not successfully sequenced.

TAP, trypsin and lipase activities were affected by dietary prebiotic incorporation only at 15 days after trial beginning, while α-amylase activity was only affected by dietary prebiotics at the end of the trial (Table 7). TAP activity was higher in fish fed FOS and XOS diets than the control diet. Trypsin activity was higher in fish fed GOS when compared with fish fed control and FOS diets while lipase activity was higher in fish fed XOS than in fish fed the control and FOS diets. α-amylase activity was lower in fish fed FOS and GOS than in fish fed control diet. All digestive enzymes activities were lower by the end of the trial than at 15 days.

4. Discussion

Prebiotics effect on fish growth, feed utilization, gut bacterial composition or digestive enzymes activities has been evaluated for some prebiotics in a few species (Dimitroglou et al., 2011; Merrifield et al., 2010a; Ringø et al., 2010, 2014). However, studies relating all of those parameters, which are important to clarify prebiotics mode of action, are very scarce (Hoseinifar et al., 2015).

As in several studies in other species, in the present study dietary scFOS, XOS and GOS incorporation had no effect on fish growth or FE (Buentello et al., 2010; Burr et al., 2010; Grisdale-Helland et al., 2008; Guerreiro et al., 2015a, b; Hoseinifar et al., 2014a, 2015). Increased FE with diets including prebiotics have however been observed in a few other studies and were associated, at least partially, to increased digestive enzymes activities that might be connected with increased exogenous microbial activity (Soleimani et al., 2012). The lack of differences on fish growth and FE in the present study may be related with the lack of prebiotics effect on gut microbiota population and digestive enzymes activities observed at the end of the trial.

In the present study, fish fed XOS had higher ALAT activity. Previous studies with turbot and gilthead sea bream (*Sparus aurata*) also reported some effect of prebiotics on amino acid catabolism enzymes, however while in turbot and effect on PER was registered, in gilthead sea bream, similarly to the present study, the differences were not reflected on PER (Guerreiro et al., 2015a, b).

It is known that prebiotics affect glucose and lipid metabolism in mammals (Delzenne et al., 2008; Gibson and Roberfroid, 1995; Roberfroid et al., 2010; Teitelbaum, 2009). In fish, however, effect of prebiotic in metabolism remains under-investigated (Guerreiro et al., 2015a, b, c; Torrecilas et al., 2011, 2015). In contrast to mammals, in the present study dietary prebiotic supplementation did not seem to improve glucose tolerance, as plasma glucose levels and the activity of key glycolytic enzymes were not different to that of the control group.

The lack of prebiotics effect on glucose metabolism may be related with the low dietary starch content (16%) which might not allowed to evidence subtle enzymatic activity changes among groups. Indeed, white sea bream is an omnivorous species and tolerates high dietary carbohydrate levels (Sá et al., 2008). In fact, an increase of GK activity due to scFOS and XOS dietary supplementation was observed in European sea bass fed FM-based diets with 20% pre-gelatinized starch, the highest dietary inclusion level recommended for carnivorous species (Enes et al., 2011; Guerreiro et al., 2015c). However, no dietary prebiotic effect on GK activity was evidenced in fish fed PF diets with 13% starch (Guerreiro et al., 2015c).

Within the prebiotics evaluated in the present study, XOS led to the lowest plasmatic triglycerides level. As reported in mammals, such effect might be related with the decrease of

Table 6
Bacterial species identified upon sequencing the DGGE bands of the gut communities of white sea bream fed the experimental diets for 15 days and at the end of the trial.

Band	Nearest neighbour	Similarity to nearest neighbour (%)	Accession number of nearest neighbour
1	Uncultured bacterium isolate DGGE gel band from jejunum, ileum and cecum of weaned piglets	96	JX183818.1
2	Uncultured *Pseudomonas* sp. from aerobic microbial fuel cell samples	95	FR870455.1
3	Uncultured bacterium isolate DGGE gel band from adult yellow grouper (*Epinephelus awoara*)	89	EU004803.1
4	Uncultured bacterium isolate DGGE gel band from a biofilm bacterial population of a chloraminated distribution system	86	EU316198.1
5	Uncultured bacterium clone thfb4-53 from eutrophic shallow lakes	84	HM050793.1
6	*Lactobacillus aviarius* subsp. *araffinosus*	99	LC071826.1
7	*Lactobacillus aviarius* subsp. *araffinosus*	98	LC071826.1
8	Uncultured *Actinobacterium* clone A46 from tomato roots	88	HM622415.1
9	*Lactobacillus gallinarum*	99	JN851792.1
10	Uncultured *Clostridium* sp. from Tinto River	90	LN680091.1
11	*Lactobacillus crispatus*	97	KM079364.1
12	Uncultured bacterium isolate DGGE gel band from sweet sorghum (*Sorghum bicolor*)	98	KC570917.1
13	Uncultured bacterium from root nodules and rhizosphere of mungbean	99	LM655381.1
14	Uncultured bacterium isolate DGGE gel band from jejunum, ileum and cecum of weaned piglets	98	JX183818.1
15	*Pseudomonas* sp. GCDBh1 from the aletsch glacier	96	GU939712.1
16	Uncultured bacterium clone ncd1673d02c1 from environmental samples	93	KF098339.1
17	Uncultured bacterium clone OYMC-Endo-CLN28 from roots of a pioneer grass (*Miscanthus condensatus*)	99	LC031369.1
18	*Pseudomonas putida*	91	KF624748.1
19	Uncultured bacterium clone JJ-84 from ramie rhizosphere soil	80	KJ782256.1

Table 7
Specific activities of digestive enzymes, total alkaline protease, trypsin, α-amylase and lipase (mU mg protein^{-1}) of white sea bream fed the experimental diets for 15 days and at the end of the trial.

Time	15 days				Final			
Diets	Control	FOS	XOS	GOS	Control	FOS	XOS	GOS
Total Alkaline Protease	259.1±12.9^{a}	310.8±40.0^{b}	313.5±37.3^{b}	301.4±42.4^{ab}	226.3±65.6	175.8±20.4	219.3±29.8	195.5±39.0
Trypsin	121.5±43.4^{ab}	108.3±33.3^{a}	168.3±36.7^{bc}	183.6±61.2^{c}	99.7±39.4	71.1±34.6	77.4±28.8	68.4±24.2
α-Amylase	529.5±198.5	504.4±162.0	529.9±116.9	705.4±346.4	441±166.8^{b}	267.0±114.1^{a}	352.6±130.0^{ab}	251.5±89.8^{a}
Lipase	4.97±0.75^{ab}	4.47±0.85^{a}	6.13±0.99^{c}	5.86±0.77^{bc}	3.80±0.74	3.02±0.54	3.20±0.90	3.26±0.79
Two-way ANOVA								
Variation source	Time	Diets	Interaction					
Total Alkaline Protease	***	ns	**					
Trypsin	***	*	**					
α-Amylase	***	ns	**					
Lipase	***	**	**					

Values presented as means ± standard deviation (n = 9). Values in the same line with different letters are significantly different (P<0.05). Two-way ANOVA: *P < 0.05; **P< 0.01; *** P < 0.001; ns: not significant.

lipogenic enzymes activity observed in this groups, and thus on *de novo* lipogenesis (Delzenne and Kok, 1999; Delzenne et al., 2002). Dietary XOS supplementation also decreased the activity of lipogenic enzymes in European sea bass, although without affecting plasmatic triglycerides levels (Guerreiro et al., 2015c).

In mammals, the decrease in lipogenesis was suggested to result from the effect of SCFAs produced during prebiotic fermentation, namely propionate and acetate (Gibson and Roberfroid, 1995; Gobinath et al., 2010; Roberfroid et al., 2010; Sangwan et al., 2015). While acetate is a lipogenic substrate, propionate is a competitive inhibitor of the protein responsible for acetate entering into hepatocytes (Delzenne et al., 2002; Teitelbaum, 2009). In fish, the presence of SCFAs, such as acetate, propionate and butyrate, due to prebiotic fermentation were also reported in *in vitro* and *in vivo* studies (Burr et al., 2008, 2010; Geraylou et al., 2012; 2013; Kihara and Sakata, 2002).

Although changes in SCFAs production are usually associated with changes in gut microbiota composition, no changes in gut bacterial communities due to dietary prebiotic supplementation were observed in this study. However, since PCR-DGGE is a semi-quantitative technique that allows the identification of the dominant microbes, the possibility of subtle changes on microbial composition that might have occurred but were not detected cannot be disregarded (Burr et al., 2010).

All prebiotics used in the current study were already reported to favour gut bacterial composition towards a more beneficial one, namely by increasing the lactic acid bacteria (LAB) community (Akrami et al., 2013; Gobinath et al., 2010; Hoseinifar et al., 2013; 2014a; 2014b; Sangwan et al., 2011; Vulevic et al., 2013). In fact, four examples of *Lactobacillus* sp., a genus of the LAB clade were found in the gut of white sea bream, though those groups were present in the gut of fish fed prebiotic and the control diet. Nonetheless, most of the identified OTUs corresponded to uncultured bacteria, as also reported in previous studies (Li et al., 2007; Merrifield et al., 2010b; Zhou et al., 2009). Three examples of *Pseudomonas* sp. were also found, which are bacteria normally isolated from fish gut (Ringø et al., 2006).

In the present study, fish were fed a PF-based diet mainly composed of soybean meal and wheat meal. It is known that both ingredients are natural sources of oligosaccharides which may have prebiotic-like properties (Aachary and Prapulla, 2011; Gibson et al., 2004). Thus, potential benefits of dietary prebiotic supplementation might have been masked. Accordingly, an absence of gut microbiota modulation due to dietary prebiotic supplementation was also observed in other fish species fed on PF-based diets (Burr et al., 2009; Dimitroglou et al., 2010; Raggi and Gatlin III, 2012).

It is assumed that feeding a prebiotic supplemented diet for 15 days is time enough to evidence potential differences in gut microbiota. It is also known that gut microbiota may change with time. For instance, mirror carp (*Cyprinus carpio*) presented differences in bacterial numbers and richness at 15 days, but in the case of the allochthonous microbiota differences disappeared at 4 weeks of feeding a functional dietary supplement (Kuhlwein et al., 2013).

Gut bacteria OTUs, richness and diversity, as well as digestive enzyme activities were lower by the end of the trial than at day 15. This may reflect an adaptation of fish to the experimental diets, as previous to the start of the trial fish were fed a FM-based diet, whereas experimental diets were PF-based. Thus, it is possible that fish gut bacteria community and digestive enzymatic activity had to adapt to cope with diet modification.

An enhancement of TAP, trypsin and lipase activities was observed in fish fed some prebiotic supplemented diets at 15 days; however by the end of the trial those differences disappeared. In red drum (*Sciaenops ocellatus*) a decrease in digestive enzymatic activity was also observed from week 4 to week 8 in all groups tested, and a transient effect of prebiotics on digestive enzymes was also reported at 4 weeks (Anguiano et al., 2013). Moreover, independently of dietary prebiotic supplementation, in the present study a relation between gut bacteria richness and diversity and digestive enzyme activities can be established at 15 days and by the end of the trial. This is in accordance with studies that reported that fish gut microbiota may have positive effects to the digestive process of fish and thus contribute to fish nutrition (Bairagi et al., 2002; Ray et al., 2012).

In conclusion, under our experimental conditions, the tested prebiotics did not affect white sea bream juveniles growth or feed utilization. Among the prebiotics tested, XOS led to a decrease of hepatic lipogenesis activity and to lower plasmatic triglycerides levels. Independently of dietary prebiotic supplementation, a relation between gut bacteria richness and diversity and digestive enzymes activities was observed. Overall, XOS seems to have good potential to be used as prebiotic in white sea bream, since an improved lipid metabolism might have the potential to produce leaner fish, which meets consumers' preference.

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Chapter 10
Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (*Diplodus sargus*)

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Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (*Diplodus sargus*)

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ABSTRACT

The aim of this study was to evaluate the effects of short-chain fructooligosaccharides (scFOS), xylooligosaccharides (XOS) and galactooligosaccharides (GOS) on immune and hepatic oxidative status, and gut morphology of white sea bream juveniles. Four diets were formulated: a control diet with fish meal (FM) and plant feedstuffs (PF) (30FM:70PF) and three test diets similar to the control but supplemented with 1% of scFOS, XOS or GOS. Dietary prebiotic incorporation did not affect total blood cell counts, hematocrit, hemoglobin, red blood indices or differential white blood cell counts. Fish fed GOS had lower ACH50 and nitric oxide than fish fed control diet. XOS enhanced immune status through the increase in alternative complement pathway (ACH50), lysozyme and total immunoglobulin. The higher activity of glucose 6-phosphate dehydrogenase in fish fed FOS compared to the other dietary groups was the only related antioxidant enzyme affected by prebiotics in the liver. GOS ameliorated the precocious adverse effects of PF based diet on gut histomorphology, as denoted by the lower incidence of histological alterations in fish fed GOS for 15 days. In conclusion, XOS and GOS at 1% might have potential to be used as prebiotics in white sea bream juveniles.

1. Introduction

Growth of aquaculture industry and intensification of production lead to increased demand of fish meal (FM) for incorporation in aquafeeds. In order to promote a sustainable aquaculture production, alternatives for FM are needed. Plant feedstuffs (PF) are nowadays the more available and used alternative to FM [1]. Though, there are still some limitations related to the use of PF in diets for fish, mainly the presence of anti-nutritional factors, such as soluble and insoluble fibers, phytic acid, enzyme inhibitors, lectins, saponins, phytosterols or oligosaccharides [2]. In fish, anti-nutrients can cause gut damages, like decrease or absence of absorptive vacuoles, shortening of heights of the mucosal foldings and infiltration of inflammatory cells in the lamina propria [3–5]. This physical barrier composed by epithelia and their mucus secretions, is part integrant and essential of fish immune system, representing one of the first lines of defence, as gut is one of the first entering routes of fish pathogens [6]. Gut-associated lymphoid tissue (GALT) in fish is formed by intraepithelial (IELs) and lamina propria (LPLs) leucocytes, including B and T lymphocytes, macrophages, and eosinophilic and neutrophilic granulocytes [7]. Therefore, damages induced in fish gut by PF might directly affect fish immune status.

Some studies in fish reported that PF reduced oxidative damage due to the presence of components with strong antioxidant activity [8–10]. In contrast, a recent study in European sea bass (*Dicentrarchus labrax*) showed that fish fed PF-based diets had higher liver lipid peroxidation levels than fish fed FM-based diets [11]. Such an effect on fish oxidative status was related with negative impacts of PF-based diets in distal intestine histomorphology. Overall, it seems that gut health, immune and oxidative status are linked.

Prebiotics are defined as non-digestible feed ingredients that promote growth and activity of beneficial bacteria present in the gut leading to improvement of host health [12]. Therefore, prebiotics are reported to modulate immune status, decrease oxidative damage or increase antioxidant potential, and improve gut morphology [13–16]. Host health improvements can be achieved

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for instance, directly, through prebiotics interaction with pattern recognition receptors, or indirectly, through increase in specific bacteria groups and the by-products produced as result of prebiotic fermentation [14,16].

Fructooligosaccharides (FOS), mannanoligosaccharides (MOS) or inulin are the most studied prebiotics in fish, while short-chain fructooligosaccharides (scFOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS) have been much less studied [15]. FOS was reported to enhance fish immunity, gut morphology and antioxidant capability in fish [17–22]. FOS and scFOS only differ on the degree of polymerization, with scFOS having only 1 to 5 fructose oligomers [23].

GOS effects on fish are scarcely studied, but available reports indicate improvement of immunity related parameters and of gut morphology [17,24]. Nonetheless, to the authors' best knowledge there is no work reporting the effect of GOS on fish oxidative status. XOS was reported to enhance fish immunity and modulate antioxidant enzymatic activities [11,25,26] but apparently has no effect on fish gut histomorphology [11,26], contrary to what was observed in rats [27].

White sea bream (*Diplodus sargus*) is a species with potential for diversification of Mediterranean aquaculture [28]. It is an omnivorous fish which tolerates relatively high levels of dietary carbohydrates [29], thus with potential for using diets with high levels of PF. A study about the potential of MOS in white sea bream larvae indicated that MOS supplementation significantly improved intestinal morphology and survival to a salinity challenge [30], this suggests that this species may be responsive to dietary prebiotics supplementation at later live stages. Therefore, the aim of the present study was to evaluate the effects of different prebiotics (scFOS, XOS and GOS) on the immune and oxidative status and gut morphology in white sea bream juveniles fed PF-based diets.

2. Material and methods

2.1. Diets composition

A control diet was formulated to contain 18% lipid and 37% protein using FM and PF (30:70 protein from FM:PF) as protein sources and fish oil as main lipid source. Three other diets were formulated identical to the control but including 1% commercial prebiotics: scFOS (PROFEED Maxflow, Jefo, France), XOS and GOS (Qingdao FTZ United International Inc., Quingdao, China), replacing α-cellulose (diets FOS, XOS, and GOS, respectively). All diet ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through 2.0 mm die. Pellets were dried in an oven at 40 °C for 48 h, and then stored in a freezer in airtight bags until use. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Chemical analyses of the diets were performed following Association of Official Analytical Chemists Methods [31]. Dietary starch content was determined according to Beutler [32].

2.2. Growth trial

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with white sea bream (*D. sargus*) juveniles obtained from IPMA, Olhão, Portugal. The trial was run in recirculating water systems equipped with 12 cylindrical fiberglass tanks of 100 L water capacity and thermo-regulated to 23.0 ± 0.5 °C. Tanks were supplied with continuous flow (2.5–3.5 L min^{-1}) of filtered seawater (35.0 ± 1.0 g L^{-1} salinity) and dissolved oxygen was kept near saturation (7 mg L^{-1}). After a quarantine period of 1 month, fish were transferred to the experimental system and adapted to the experimental conditions for 15 days. Thereafter, 19 white sea bream with an initial mean body weight of 53.0 ± 0.03 g were distributed to each tank and the experimental diets randomly assigned to triplicate groups. The trial lasted 12 weeks and during that period fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. The experiment was performed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Table 1
Ingredients and proximate composition of the experimental diets.

	Diets			
	Control	FOS	XOS	GOS
Ingredients (% dry weight)				
Fish meal[a]	15.4	15.4	15.4	15.4
Corn gluten[b]	13.3	13.3	13.3	13.3
Wheat meal[c]	25.1	25.1	25.1	25.1
Soy meal[d]	25.0	25.0	25.0	25.0
Cod liver oil	15.1	15.1	15.1	15.1
Bicalcium phosphate[e]	1.6	1.6	1.6	1.6
Fructooligosaccharide[f]	–	1.0	–	–
Xylooligosaccharide[g]	–	–	1.0	–
Galactooligosaccharide[g]	–	–	–	1.0
α-Cellulose[h]	1.0	–	–	–
Vitamin mix[i]	1.0	1.0	1.0	1.0
Mineral mix[j]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder (aquacube)[k]	1.0	1.0	1.0	1.0
Proximate analysis (% dry weight)				
Dry matter (%)	90.7	88.4	91.7	88.8
Crude protein	37.2	37.7	36.9	37.3
Crude fat	18.7	18.6	18.4	18.2
Ash	8.1	7.9	8.7	8.1
Starch	16.1	15.5	16.2	16.5
Gross energy (kJ g^{-1})[l]	18.9	18.9	18.8	18.8

DM: dry matter; CP: crude protein; GL: gross lipid.
[a] Steam Dried LT fish meal, Pesquera Diamante, Austral Group, S.A Perú (CP: 71.2% DM; GL: 9.1% DM).
[b] Sorgal, S.A. Ovar, Portugal (CP: 67.4% DM; GL: 2.7% DM).
[c] Sorgal, S.A. Ovar, Portugal (CP: 10.9% DM; GL: 2.8% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 54.1% DM; GL: 1.9% DM).
[e] Premix, Portugal (Calcium: 24%; Total phosphorus: 18%).
[f] scFOS: PROFEED Maxflow, Jefo, France.
[g] Quingdao FTZ United International Inc., Quingdao, China.
[h] Sigma-Aldrich, Sintra, Portugal.
[i] Vitamins (mg kg^{-1} diet): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400.
[j] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).
[k] Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate).
[l] Gross energy calculated based on theoretical values (CP: 23.6 kJ g^{-1}; GL: 39.5 kJ g^{-1}; Carbohydrates: 17.2 kJ g^{-1}).

2.3. Sampling

Fifteen days after the start of the feeding trial, 3 fish from each tank were randomly sampled 3 h after the morning meal and anesthetized by immersion in 0.3 ml L^{-1} ethylene glycol monophenyl ether [33]. Blood was collected from the caudal vein using heparinized syringes, placed in heparinized tubes and centrifuged at 3000 × g for 10 min at room temperature. The resulting plasma was frozen at −80 °C for immune parameters analysis (n = 9 for

each diet). After blood collection, fish were euthanized with a sharp blow in the head, dissected on chilled trays and the digestive tract was freed from adjacent adipose and connective tissues. Two pyloric caeca (PC) and a section of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) were sampled for histological evaluation (n = 9 for each diet). The samples were rinsed in phosphate buffered saline (PBS), carefully blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%, pH 7.4) for 24 h and subsequently transferred to ethanol (70%) until further processing.

At the end of the trial, 3 fish per tank were randomly sampled according to the first sampling procedure. Blood and intestine were collected as described above (n = 9 for each diet). Blood was also used for hematological assessment (n = 9 for each diet). Livers were sampled from the same fish, immediately frozen in liquid nitrogen and stored at −80 °C until measurement of enzymes activities and lipid peroxidation levels (n = 9 for each diet).

2.4. Hematological analysis

Fresh heparinized blood was used for hematocrit (Ht) and hemoglobin (Hb) determination and blood cells counts. Ht, Hb, total red blood cells (RBC), white blood cells (WBC) and differential WBC counts were determined as described by Guerreiro et al. [34].

2.5. Immune parameters

Alternative complement pathway (ACH50), nitric oxide (NO), anti-protease, lysozyme and peroxidase activities and total immunoglobulins (Ig) in plasma were determined as described in Guerreiro et al. [34] and Machado et al. [35].

2.6. Enzyme activity

Liver samples were homogenized on ice in five volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. Homogenates were centrifuged at 30,000 × g for 30 min at 4 °C, and the resultant supernatants were separated in aliquots and stored at −80 °C until use. All enzyme assays were carried out at 37 °C in a Multiskan GO microplate reader (Model5111 9200; Thermo Scientific, Nanjing, China). The optimal substrate and protein concentrations for measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 mM^{-1} cm^{-1}, respectively. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) and glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) activities were determined as described by Guerreiro et al. [34]. Protein concentration in the homogenates was determined by the Bradford method [36] using Sigma protein assay kit (ref. B6916) with bovine serum albumin as a standard.

Enzyme activity was expressed as units (SOD and CAT) or milliunits (GPX, GR and G6PD) per mg of hepatic soluble protein. Except for SOD, one unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the assay conditions. One unit of SOD activity was defined as the amount of enzyme necessary to produce 50% inhibition of the ferricytochrome c reduction rate.

2.7. Lipid peroxidation (LPO)

Malondialdehyde (MDA) concentration was used as marker of LPO level in the liver. In the presence of thiobarbituric acid, MDA reacts producing colored thiobarbituric acid reacting substances (TBARS) that were measured as described by Guerreiro et al. [34]. Results were expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

2.8. Histological processing and morphological evaluation

PC and DI samples were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Blind evaluation of histological preparations was performed with particular attention given to any inflammatory changes [4,37], namely changes observed in mucosal folds height (FH), width and cellularity of the lamina propria (LP) and submucosa (SM), number of intraepithelial lymphocytes (IELs), numbers of eosinophilic granulocytes (EGCs), nucleus position within the enterocytes (ENT) and size and variation of enterocyte vacuolization (SNV). A scale scoring system was used as described by Penn et al. [38] with the range of tissue scores set from 0 (normal) to 5 (highly modified). The overall value of histomorphological alterations was calculated by averaging scores of the parameters described above. Images were acquired with Zen software (Blue edition; Zeiss, Jena, Germany).

2.9. Statistical analysis

Data are presented as means ± standard deviation. Statistical analysis of the immune humoral parameters was done by two-way ANOVA, with diets and sampling time as factors. Data were tested for normality by the Shapiro–Wilk test and for homogeneity of variances by the Levene test. When normality was not verified, data was transformed prior to ANOVA. Significant differences among groups were determined by the Tukey multiple range test. Histological data was neither normal nor homogeneous and could not be normalized, thus the Kruskal–Wallis non-parametric test and subsequent pairwise comparison was performed. All other data was analyzed by one-way ANOVA. The probability level of 0.05 was used for rejection of the null hypothesis. All the statistical analysis was done using the SPSS 22 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

Dietary prebiotic incorporation did not affect total blood cell counts, Ht, Hb, RBC indices nor differential WBC counts (Table 2).

Independently of diet composition, NO, lysozyme and peroxidase activities were higher at the end of the trial, while anti-protease activity and total Ig were higher 15 days after the beginning of the trial (Table 3). ACH50, lysozyme and total Ig were higher in fish fed XOS diet than control diet. ACH50 and NO were lower for fish fed GOS than in fish fed the control diet (Table 3).

Except for G6PD, antioxidant enzymatic activity and LPO levels were not affected by dietary prebiotics (Fig. 1). G6PD activity was higher in fish fed FOS diet.

Mean scores of PC and DI at 15 days and by the end of the trial, representing the average of the separate parameters evaluated (FH, LP, SM, IELs, EGCs, ENT and SNV) are presented in Table 4. PC was not affected by dietary prebiotic incorporation neither at 15 days not at the end of the trial. DI of fish fed GOS had a lower score than fish fed control diet 15 days after the start of the trial. By the end of the trial, however, no differences between dietary groups was observed. The higher mean score obtained in the DI of control group after 15 days is related to increased alterations of enterocytes general architecture and vacuolization (Fig. 2).

Table 2
Hematological parameters and differential white blood cell counts of white sea bream fed the experimental diets for 12 weeks.

Diets	Control	FOS	XOS	GOS
RBC ($\times 10^6$ mm^{-3})	2.04 ± 0.52	1.67 ± 0.52	1.99 ± 0.46	1.95 ± 0.52
Ht (%)	24.9 ± 3.1	23.8 ± 3.3	25.9 ± 3.8	24.9 ± 5.9
Hb (g dl^{-1})	5.62 ± 0.68	5.04 ± 0.80	5.56 ± 1.01	5.43 ± 1.28
MCV (μm^3)[a]	129.4 ± 35.8	130.0 ± 16.5	134.9 ± 31.0	124.0 ± 30.1
MCH (pg cell^{-1})[b]	0.79 ± 0.17	0.76 ± 0.11	0.79 ± 0.22	0.74 ± 0.19
MCHC (g 100 mL^{-1})[c]	0.62 ± 0.06	0.64 ± 0.08	0.58 ± 0.05	0.60 ± 0.06
WBC ($\times 10^4$ mm^{-3})	3.80 ± 0.87	5.00 ± 1.84	4.86 ± 1.26	4.52 ± 1.47
Thrombocytes ($\times 10^4$ mm^{-3})	0.95 ± 0.30	1.52 ± 0.78	1.67 ± 0.92	1.15 ± 0.66
Lymphocytes ($\times 10^4$ mm^{-3})	2.61 ± 0.92	3.17 ± 1.48	2.94 ± 0.99	3.07 ± 1.03
Monocytes ($\times 10^4$ mm^{-3})	0.11 ± 0.05	0.15 ± 0.21	0.12 ± 0.06	0.13 ± 0.09
Neutrophils ($\times 10^4$ mm^{-3})	0.13 ± 0.11	0.16 ± 0.21	0.13 ± 0.08	0.17 ± 0.09

Values presented as means ± standard deviation (n = 9). No significant differences were observed between dietary treatments (P > 0.05).
RBC: red blood cell counts; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cell counts.
[a] MCV: (Ht/RBC) × 10.
[b] MCH: (Hb/RBC) × 10.
[c] MCHC: (Hb/Ht) × 100.

Table 3
Immune humoral parameters of white sea bream fed the experimental diets for 15 days and at the end of the trial (12 weeks).

		Diets			
	Time	Control	FOS	XOS	GOS
ACH50	15 days	b 71.2 ± 11.5	ab 58.8 ± 7.9	c 84.0 ± 19.2	a 57.1 ± 6.5
	12 weeks	b 60.2 ± 13.2	ab 62.4 ± 18.0	c 80.2 ± 17.1	a 46.8 ± 10.5
Nitric oxide (μM)	15 days	b 58.7 ± 28.1	ab 48.5 ± 19.0	ab 53.0 ± 15.6	a 47.9 ± 17.8 A
	12 weeks	b 301.8 ± 35.6	ab 256.8 ± 65.9	ab 229.7 ± 63.1	a 221.9 ± 65.5 B
Anti-protease activity (%)	15 days	86.6 ± 3.8	87.0 ± 1.2	88.0 ± 0.8	86.7 ± 1.3 B
	12 weeks	86.4 ± 1.3	86.1 ± 4.0	85.6 ± 2.9	82.9 ± 3.3 A
Lysozyme (mg ml^{-1})	15 days	a 1.02 ± 0.82	ab 1.97 ± 0.65	b 2.07 ± 0.79	a 1.69 ± 0.85 A
	12 weeks	a 2.32 ± 0.59	ab 2.33 ± 1.60	b 4.08 ± 2.64	a 1.81 ± 1.05 B
Peroxidase (U ml^{-1})	15 days	204.8 ± 64.2	306.0 ± 203.0	234.3 ± 169.9	176.9 ± 68.1 A
	12 weeks	455.3 ± 168.2	344.8 ± 171.1	336.7 ± 164.6	352.6 ± 170.8 B
Total immunoglobulin (mg ml^{-1})	15 days	ab 25.1 ± 6.2	a 21.4 ± 6.8	c 31.7 ± 8.9	bc 32.1 ± 8.1 B
	12 weeks	ab 23.1 ± 7.2	a 17.6 ± 5.7	c 32.6 ± 10.2	bc 22.8 ± 5.6 A

Values presented as means ± standard deviation (n = 9).
Means with different lower case letters denotes for significant differences between diets, while means with different capital letters denotes for significant differences between sampling times (Two-way ANOVA; Tukey test, p < 0.05). To facilitate visualization, capital letters are represented only in GOS diets, but those letters represent the statistics of all diets.

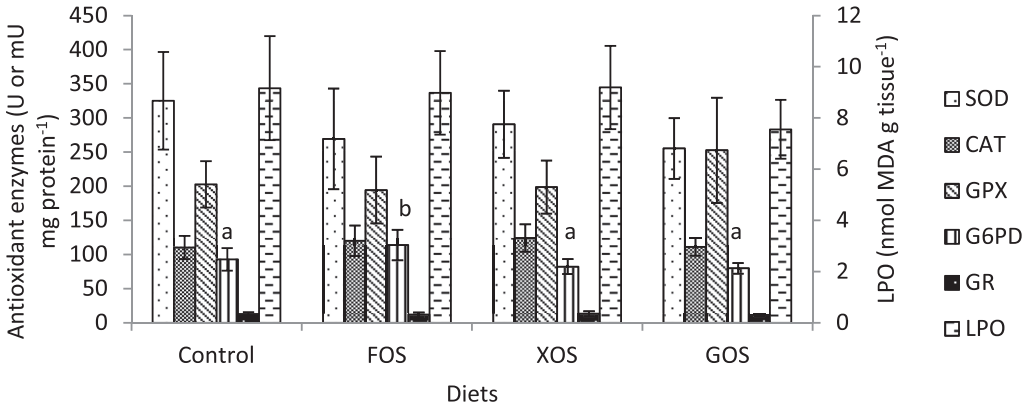

Fig. 1. Specific activities of hepatic antioxidant enzymes and lipid peroxidation (LPO) levels of white sea bream fed the experimental diets for 12 weeks. Values presented as means ± standard deviation (n = 9). Superoxide dismutase (SOD) and catalase (CAT) are expressed as U mg protein^{-1}. Glutathione peroxidase (GPX), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PD) are expressed as mU mg protein^{-1}. LPO is expressed as nmol malondialdehyde (MDA) g tissue^{-1}. Bars with different letters are significantly different (P < 0.05).

4. Discussion

Studies generally report enhanced immune status in fish due to prebiotic supplementation. Caspian roach (*Rutilus rutilus*) fed 1, 2 or 3% FOS during 7 weeks presented increased Ig, lysozyme and ACH50 [18]. Red drum (*Sciaenops ocellatus*) fed 1% FOS, MOS, transgalactooligosaccharide and GroBiotic®-A during 4 weeks or turbot (*Scophthalmus maximus*) fed 0.04% XOS during 72 days had increased values of lysozyme [24,25]. Gilthead sea bream (*Sparus aurata*) fed 1% inulin during 4 weeks showed higher values of

Table 4
Intestinal histology of pyloric caeca (PC) and distal intestine (DI) of white sea bream fed the experimental diets for 15 days and at the end of the trial (12 weeks). Mean scores were calculated by averaging the scores of the separate parameters evaluated (changes observed in mucosal folds height, width and cellularity of the lamina propria and submucosa, number of intraepithelial lymphocytes, numbers of eosinophilic granulocytes, nucleus position within the enterocytes and size and variation of enterocyte vacuolization).

		Diets			
	Time	Control	FOS	XOS	GOS
PC	15 days	1.70 ± 0.22	1.56 ± 0.23	1.50 ± 0.24	1.52 ± 0.32
	12 weeks	1.63 ± 0.20	1.88 ± 0.38	1.60 ± 0.20	1.72 ± 0.34
DI	15 days	1.76 ± 0.28 b	1.70 ± 0.16 ab	1.62 ± 0.37 ab	1.48 ± 0.13 a
	12 weeks	1.62 ± 0.17	1.51 ± 0.26	1.47 ± 0.29	1.36 ± 0.27

Values presented as means ± standard deviation (n = 9). Score from 0 to 5, with 5 indicating major alterations.
Values in the same line with different letters are significantly different ($P < 0.05$).

suggested to occur in Siberian sturgeon fed AXOS [40].

Dietary GOS effects on fish immunity are scarce and contradictory [17,47,48]. Hoseinifar et al. [48] in rainbow trout (*Oncorhynchus mykiss*) reported increased lysozyme, ACH50 and respiratory burst activities, while Grisdale-Helland et al. [47] in Atlantic salmon (*Salmo salar*) reported a lack of effect on blood neutrophil oxidative radical production and a trend towards lower lysozyme activity. In the present study, GOS supplementation lead to a reduction of ACH50 and nitric oxide (NO), which suggest that GOS might decrease phagocytic activity, since NO is a product of activated macrophages [49]. More studies are needed to elucidate GOS effect on fish immune status, namely in the decrease of immune parameters levels, such as the ACH50 and NO registered in the present study.

Although it was stated that there are no specific cellular FOS

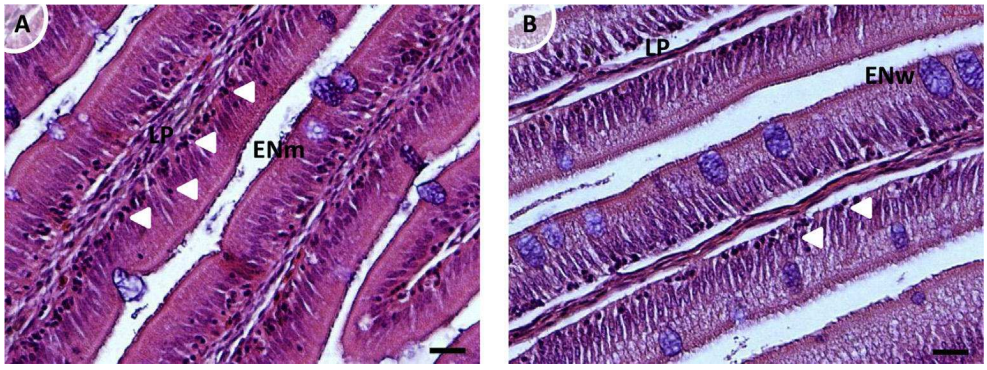

Fig. 2. Detail of relevant alterations found in the distal intestine of white sea bream fed control (A) and GOS (B) diets for 15 days. (lamina propria: LP; intraepithelial leukocytes: white arrow heads; enterocytes showing slightly misaligned nucleus and decreased vacuolization: ENm; enterocytes showing well align nucleus and homogenous vacuolization: ENw) Scale bar: 20 μm; H–E staining.

complement and IgM [39]. Siberian sturgeon (*Acipenser baerii*) fed 2% arabinoxylan-oligosaccharides (AXOS) during 12 weeks presented increased ACH50 [40]. Concerning hematological parameters, stellate sturgeon (*Acipenser stellatus*) fed 1 or 2% FOS and beluga (*Huso huso*) fed 1% inulin exhibited higher WBC counts [19,41]. In the latter species, an increase of lymphocytes (%) was observed when fish was fed 1 and 2% oligofructose and increased Ht in fish fed 2% oligofructose [42].

In the present study and in line with the unchanged WBC counts, as well as the other hematological parameters assessed, an absence of prebiotics effect on anti-protease and peroxidase was also observed. However, lysozyme and ACH50 were higher in fish fed XOS than the control diet. This might indicate higher capacity of fish to respond against bacterial infections. Further, as ACH50 disrupts the outer membrane of bacteria cell allowing lysozyme to gain access to the cell [43], the increase of both parameters in fish fed XOS acts synergistically to potentiate innate immune defenses of fish.

Total Ig was also higher in fish fed diets supplemented with XOS, suggesting that XOS might actively stimulate the secretion of IgM by B-cells as also observed in gilthead sea bream fed inulin [39]. The immunostimulatory nature of XOS may be a direct effect of the prebiotic itself on pattern recognition receptors, such as the β-glucan receptors and dectin-1 receptors, or an indirect effect, related to stimulation of gut beneficial bacteria growth [16,24,40,44] which may have immunostimulatory properties [45,46]. Additionally, short-chain fatty acids (SCFAs) produced as result of prebiotic fermentation by bacteria, could be taken up by the host contributing to enhance the immune status, as it was receptors on vertebrate cells [14] several recent studies reported an enhancement of immune parameters in fish fed FOS [17–19,21,22]. Hence, red drum, Caspian roach, stellate sturgeon, triangular bream (*Megalobrama terminalis*) and common carp (*Cyprinus carpio*) presented increased values of for instance WBC, lysozyme, ACH50 or Ig. The mechanism beyond those effects in fish is still to be completely elucidated. However, in humans FOS was reported to interact with toll-like receptor 2, a membrane protein receptor, present on macrophages, polymorphonuclear leukocytes, granulocytes and dendritic cells, which lead to immune cell activation through signal transduction pathways [50]. Nonetheless, in the present study dietary FOS did not affect fish immune parameters measured, as it was also the case in other studies [34,47,51].

Long term administration of immunostimulants to fish has been questioned by some authors, since may induce immunosuppression [47,52,53]. In the present study, the effect of XOS on the humoral immune parameters apparently was not affected by time, showing that no immunosuppression was caused by dietary prebiotics, since differences between control fish and treatment fish were the same at 15 days and at the end of the trial.

During phagocytosis macrophages and neutrophils produce reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl free radical, in a mechanism called respiratory burst [43,54–56]. Besides respiratory burst, free radicals and ROS are continuously being generated as products of cell oxidative metabolism in all aerobic organisms, including fish [57]. ROS are highly bactericidal due to their oxidizing activity [43,54]. However, when ROS production is higher than ROS removal oxidative stress occurs. Fish as other aerobic organisms are also susceptible to ROS

attack and developed antioxidant defenses based on substances such as vitamins C and E, uric acid, glutathione, and carotenoids, besides a complex antioxidant enzymes system [58,59]. In the present study, lipid peroxidation levels and antioxidant enzymes activities suggested that dietary prebiotic supplementation had no effect on ROS production in the liver.

Indeed, only G6PD activity was higher in fish fed FOS, none of the other measured antioxidant enzymes activities were affected by diet. G6PD generates NADPH which is essential for the activity of the antioxidant enzymes CAT, GPX and GR [58,60] but also for the production of NADPH necessary for fatty acid synthesis. Thus, the higher activity of G6PD might be connected with the glycolytic and lipogenic metabolic pathways. In fact, glucose and lipid metabolism modulation due to prebiotic dietary supplementation was already reported in mammals and fish [61–63].

It is well known that the anti-nutritional factors presented in PF, namely those present in soybean meal, can cause moderate and severe enteritis in fish [3,5]. In the present study, although no overt inflammation was observed, a beneficial effect of GOS dietary incorporation was observed in DI histomorphology after 15 days of feeding fish with PF-based diets. Improvements in fish gut histomorphology due to dietary prebiotic supplementation were also reported for other fish species fed on PF-based diets [17,64–66]. However, red drum, gilthead sea bream, Atlantic salmon and European sea bass are all mainly carnivorous while white sea bream is omnivorous. Prebiotics interact directly with enterocytes, since SCFAs produced during prebiotic fermentation by gut bacteria are absorbed and used as energy source by enterocytes [14,67]. European sea bass fed PF-based diets supplemented with MOS presented a more preserved cytoarchitecture of the intestinal epithelial barrier, improvements that were suggested to be achieved by increased polar lipids and prostaglandins levels, and consequent increased mucus production [66]. In the present study GOS might have acted at the same level, since enterocyte general morphology and vacuolation were more close to normal in GOS group; nevertheless more studies are needed to fully elucidate such effect. However, at the end of the trial no differences were observed between control and prebiotic treatments suggesting that fish were able to adapt to the high dietary PF levels, thus masking prebiotics effects. These results are somewhat expectable and might be related to white sea bream omnivorous feeding habits.

In conclusion, the tested prebiotics affected fish immune parameters differently. XOS stimulated ACH50, lysozyme and total Ig, indicating a possible enhanced fish immune status. To be sure of the capabilities of fish to survive to an infection, challenge trials should be thought in future studies. GOS seemed to decrease fish immune response while a lack of effect was observed with FOS. Prebiotics had no major effect on fish oxidative status in the liver. GOS reduced gut histomorphology alterations during the initial period of feeding with PF-based diets. XOS and GOS at 1% might have potential to be used as prebiotics at least in white sea bream juveniles, but since those are prebiotics understudied more studies are needed to clarify their specific action mechanisms.

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Chapter 11
General conclusions and final considerations

11.1 General conclusions

The results of the present thesis allowed to formulate the following conclusions:

- Rearing temperature and prebiotic dosage may affect fish response to prebiotics, namely scFOS (Chapter 2, Chapter 3, Chapter 4, Chapter 5, and Chapter 6).
- Temperature affected gut microbiota in turbot, with higher temperature (15°C compared to 20°C) increasing bacterial richness and diversity (Chapter 3). On the contrary, in gilthead sea bream rearing temperature (18°C and 25°C) did not affect bacterial richness and diversity (Chapter 6). This seems to indicate that temperature effect on gut microbiota composition may be species dependent.
- Comparatively to optimal rearing temperature (15°C), high temperature (20°C) does not seem to increase hepatic oxidative stress in turbot (Chapter 4).
- scFOS seems to affect turbot's oxidative stress response, but effects were temperature related (Chapter 4).
- scFOS had no remarkable effect in overall fish performance (Chapter 2, Chapter 5, Chapter 7, and Chapter 9).
- XOS increased European sea bass growth performance in PF-based diets (Chapter 7); however, in white sea bream no effects of XOS were observed in growth performance (Chapter 9).
- A positive correlation between dietary scFOS incorporation and fish growth was observed at 15°C in turbot (Chapter 2) and at 18°C in gilthead sea bream (Chapter 5). This seems to indicate a possible beneficial effect of dietary scFOS incorporation when fish are reared at low temperatures.
- No prebiotics effect in gut microbiota communities were detected by PCR-DGGE (Chapter 3, Chapter 6 and Chapter 9).
- In line with mammals, XOS decreased lipogenesis in European sea bass and white sea bream (Chapter 7 and Chapter 9).
- In European sea bass fed FM diets, XOS and scFOS increased glycolytic activity (Chapter 7).
- PF-based diets increased European sea bass liver lipid peroxidation levels and had negative impacts in the distal gut histomorphology compared with fish fed FM-based diets (Chapter 8).
- XOS incorporation, both in PF and FM diets fed to European sea bass, reduced antioxidant enzymatic activity, suggesting a role in the reduction of ROS production (Chapter 8).
- scFOS and XOS incorporation in PF diets were not effective in counterbalancing the negative effects of PF diets in gut morphology of European sea bass (Chapter 8).
- In white sea bream, GOS ameliorated the histomorphological alterations that occurred during the initial period (15 days) of feeding with PF-based diets (Chapter 10).

- In white sea bream, XOS stimulated some of the measured immune system parameters, indicating that it may contribute to an enhanced fish immune status (Chapter 10).

Summarizing, XOS improved growth performance in fish fed PF-based diets. As reported in mammals, XOS also decreased lipogenesis in European sea bass and white sea bream. XOS effect on lipogenesis of European sea bass and white sea bream suggest that its effect on intermediary metabolism may be similar among species. In white sea bream, XOS stimulated some immune parameters. Thus, from the three prebiotics tested (scFOS, XOS, and GOS), XOS seems the most promising to be used in fish aquaculture, namely in diets rich in PF ingredients.

Dietary scFOS may also have potential in improving fish growth when fish are reared at low temperatures.

11.2 Final considerations

One of the main conclusions of the present thesis is that XOS seems a promising prebiotic to be used in fish aquaculture. Therefore, this prebiotic should be further studied in other fish species and combining other study areas. Moreover, the fact that XOS seemed to have the same effect in a carnivorous and an omnivorous fish, increases the need of checking that in other fish species.

Assuming that prebiotics act directly in fish gut microbiota, and since in the current thesis no effect was detected in gut microbiota communities using PCR-DGGE, a semi-quantitative technique, quantitative techniques such as fluorescent in situ hybridization, quantitative real-time PCR and next-generation sequencing should be used in future studies as a means to overcome limitations associated with PCR-DGGE (Rastogi and Sani, 2011; Zhou et al., 2014). Although PCR-DGGE is a sensitive technique, which allows identification of the dominant microbes present in environmental samples, it also presents some problems. PCR-DGGE uses a low number of nucleotides in primers, which difficult resolution between highly similar strains and the identification beyond genus level can be difficult. Moreover, PCR amplicons with similar denaturing characteristics may migrate to the same location lane, complicating gel interpretation and operational taxonomic units identification (Zhou et al., 2014).

Since prebiotics beneficial effects may be related to changes in microbial community activities instead of changes in numbers or diversity, future studies about SCFAs production (Gibson and Roberfroid, 1995) should be though when testing prebiotics effects.

Most studies that tested prebiotics effect against bacterial or stress challenges reported improved fish resistance (Cerezuela et al., 2012; Hoseinifar et al., 2013; Hoseinifar et al., 2014b; Hoseinifar et al., 2015c; Soleimani et al., 2012; Torrecillas et al., 2007; Torrecillas et al., 2012). Although prebiotics affect immune related parameters or fish oxidative stress status, to further elucidate these potential effects challenge trials should be made. The most promising

and practical challenges are the ones involving bacteria commonly responsible for heavy losses in aquaculture production, or changes in environmental conditions, such as sudden temperatures changes, which may be a problem in the aquaculture industry.

Recently, some authors started to unravel the mechanisms beyond prebiotic effects on fish (Cerezuela et al., 2012; Cerezuela et al., 2013b; Torrecillas et al., 2015b). However, those studies are still in their early stages and much work still needs to be performed, as most knowledge still originates from mammal models. Thus, studies about the expression of genes regulating the observed prebiotics effects are necessary and of utmost importance.

Contrary to the reported decrease in lipogenesis in mammals fed FOS (Delzenne, 2003; Gibson and Roberfroid, 1995; Roberfroid et al., 2010), the tendency for lipogenesis increase with scFOS dietary incorporation observed in this thesis should be further confirmed.

Finally, the observed beneficial effect of scFOS at low rearing temperatures deserves to be further exploited. Maybe in some fish, scFOS beneficial effects in growth are only triggered when fish are reared in non-perfect conditions, as in the case of gilthead sea bream reared at low temperatures. Since, during winter, outdoor aquacultures might face very low rearing temperatures this is a field that deserves to be further explored.

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